University of Alberta

The Regulation of Histone Acetylation through Nuclear Compartmentalization

by

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This thesis is dedicated in loving memory of my father Sandy MacLean.
As I once lived in his dreams and became a reality in his life so may he live in and guide my dreams through my life.

I. ABSTRACT

The objective of this study was to examine the functional significance of nuclear compartmentalization of histone acetyltransferases (HATs) and histone deacetylases (HDACs) with respect to transcriptional regulation. To critically test the hypothesis that controlled nuclear localization is involved in the regulation of histone acetylation and chromatin remodeling, I used cells infected with the Herpes Simplex Virus 1 (HSV-1). During infection, several histone acetyltransferases and deacetylases are recruited to viral replication compartments in the nucleus and the timing of the recruitment correlates with the general repression of host gene transcription by RNA Polymerase II. Recruitment also correlates with a loss of transcription-associated highly acetylated histone epitopes on host chromatin. I hypothesize that transcription-associated histone acetylation is repressed during HSV-1 infection through an alteration of the steady-state distributions of enzymatic machinery rather than catalytic inactivation. Data is presented showing that histone acetylation is reduced in virally infected cells and this correlates temporally with the recruitment of HATs and HDACs to viral replication compartments. The nature of HATs' and HDACs' mobility within and outside of viral replication compartments is characterized, showing a shift in steady-state distribution favoring association with viral compartments rather than irreversible binding within these compartments.

II. ACKNOWLEDGEMENT

Truth is one, paths are many.

This is perhaps the most valuable lesson learned through the two years of research to produce the body of work presented in this Masters thesis and the seemingly infinite time to produce this document, which comes only as a result of time spent for deep personal growth and development. I started down this path with my supervisor; Dr. Michael Hendzel, and it is with a deep sense of gratitude that I wish to extend my sincere thanks for giving me the opportunity to hone my ambition and enthusiasm for the unknown to develop as a critical thinker, academically and otherwise. Despite not always seeing eye-to-eye, I delighted to find that when our eyes did meet, we would often crack a smile. Michael's company, assurance and understanding of my efforts to help my father, and indeed myself, during his long illness are why I am forever thankful to him. I have learned so much by being kept in the dark (of the confocal room that is). I would have beer for breakfast with him any day.

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VI. LIST OF ABBREVIATIONS

AP-2 – activator protein-2

ATM – Ataxia-Telangiectasia mutated

AUT – acid urea triton X-100

BrDU – 5-Bromo-2' deoxy-uridine

$CaCl_2$ – calcium chloride

CBP – cyclic AMP response element binding protein binding protein

CoA – coenzyme A

CpG – cytosine-phosphate-guanine

CREB – cyclic AMP response element binding

CREM – cyclic AMP response element modulator

DAPI – 4',6-diamidino-2-phenylindole

ddH_2O – distilled deionized water

DIM – digital imaging microscope

DMEM - Dulbecco's Modification of Eagle's Medium

DNA – deoxyribonucleic acid

dpm – disintegrations per minute

DTT – dithiothreitol

E genes – early gene

EDTA – ethylenediaminetetraacetic acid

FAT – factor acetyltransferase

FLIP – fluorescence lost in photobleaching

FRAP – fluorescence recovery after photobleaching

GFP – green fluorescent protein

HAc – acetic acid

HAT – histone acetyltransferase

HCl – hydrochloric acid

HDAC – histone deacetylase

HeNe laser – helium neon laser

HEPES - N-(2-hydroxyethyl)piperazine-N’-(2- ethanesulfonic acid)

HISM – human intestinal smooth muscle

HSV – Herpes Simplex Virus

ICG – interchromatin granule cluster

IE gene – immediate early gene

IgG – immunoglobin G

IM – Indian Muntjac

IR – infared

K – lysine

KCl – potassium chloride

L gene – late gene

$MgCl_2$ – magnesium chloride

MOI – multiplicity of infection

MYST – MOZ, Ybf2/Sas3, Sas2 and Tip60

N.A. – numerical aperture

NaCl – sodium chloride

NCoR – nuclear receptor corepressor

NuMA – nuclear mitotic apparatus

NuRD – nucleosome remodeling and histone deacetylation complex

PBS – phosphate buffered saline

PCAF – p300/CBP associated factor

PKA – protein kinase A

PML – promyelocytic leukemia

PMSF – phenylmethyl sulfonylfluoride

pRb – retinoblastoma protein

RNA – ribonucleic acid

RNAP – RNA Polymerase

SD – standard deviation

SDS-PAGE – sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SFC – splicing factor compartment

SMRT – silencing mediator for retinoic acid and thyroid hormone receptor

TAF – TATA-binding protein-associated factor

TBP – TATA-binding protein

TEMED - N,N,N,N'-tetramethylethylenediamine

Ti:Sapphire – titanium sapphire

TSA – Trichostatin A

UTP – uradine 5'-triphosphate

UV - ultraviolet

VP – viral protein

VRC – viral replication compartment

1. INTRODUCTION

1.1 Overview

Over the past three decades, research into eukaryotic gene transcription has revealed a remarkably intricate biochemical process that is orchestrated on many levels. Early findings elucidated the natures of transcriptionally active euchromatin and transcriptionally inactive heterochromatin, which were found to differ primarily by the high or low acetylation state of the core histones respectively (Allfrey *et al.*, 1964; Allfrey, 1966; Sealy and Chalkley, 1978; Clayton *et al.*, 1993). This view of regulation expanded to include findings such as the identification of upstream regulatory sequences, the isolation of sequence-specific DNA binding proteins that displayed unique properties, functions, and protein-protein associations, and research concerning the enzymatic modulation of chromatin structure itself to better accommodate these factors (Lemon and Tjian, 2000). Histone acetyltransferases (HATs) and deacetylases (HDACs) were identified as the enzymes responsible for this alteration of chromatin ultrastructure by the covalent acetylation and deacetylation of specific lysine residues on core histone tails (Gray and Ekström, 2001; Roth *et al.*, 2001; de Ruijter *et al.*, 2003). This led to the characterization of the assembly and function of multisubunit complexes that were termed transcriptional coactivators and corepressors, most possessing HAT and HDAC activities respectively (Brown *et al.*, 2000; Burke and Baniahmad, 2000; Cress and Seto, 2000; Näär *et al.*, 2001). Naturally, the study of these and other functionally specialized nuclear factor complexes led to questions addressing the mechanism of the formation of these complexes and the nuclear localization of their individual and complexed component populations within the nuclear space.

Historically, insight into the nature of a dynamic but functionally compartmentalized nucleus as compared to other organelles within the cell was challenging as the nucleus does not lend itself easily to biochemical analysis. However, with the development of advanced microscopy and molecular techniques, evidence has begun to accumulate for an additional level of transcriptional regulation. This regulation operates at the level of subnuclear organization of functionally distinct chromatin domains and the factors within the interchromatin space (Lamond and Earnshaw, 1998; Cremer and Cremer, 2001; Belmont, 2003). Understanding the control of the subnuclear distribution of regulatory proteins may be fundamental to the spatial and temporal regulation of gene transcription, and the final aspect needed to fully elucidate the mechanism of transcriptional regulation. This underscores the importance of the study of a global functional organization of the nucleus.

A powerful method used to study the functional significance of nuclear organization without compromising the ability of proteins to form compartments is to impose a system that globally influences and disrupts normal subnuclear organization. Nature has provided a means to do this via the infection of eukaryotic cells with Herpes Simplex Virus-1 (HSV-1). HSV-1 establishes distinct viral replication compartments in the host nucleus that exclude host chromatin without employing physical barriers such as membranes (Puvion-Dutilleul *et al.*, 1985; Spencer *et al.*, 2000). While the virus encodes proteins sufficient for viral replication, for gene expression it must utilize the host transcriptional and RNA processing machinery through the recruitment of RNA Polymerase II (RNAPII) and other factors (Godowski and Knipe, 1986; Weinheimer and

McKnight, 1987). The multifactorial nature of RNAPII transcription initiation does not allow for a simple interpretation of these results, as either certain factors needed for host transcription are preferentially recruited so active complexes cannot form or, although complexes may be present, the altered spatial rearrangement of the complexes do not make them available to the host chromatin.

Histone acetylation is an ideal functional model for nuclear compartmentalization. It has been shown by indirect immunofluorescence microscopy that HATs and HDACs are localized primarily in the nucleus (Hendzel *et al.*, 2001). While their activity is essential to transcriptional activation, it is not, like transcription, dependent upon accessory proteins. Both the substrate and products are known and available for proven activity assays, and selective inhibitors of HDACs are available to measure ongoing, rather than simply steady-state, acetylation and deacetylation (Emiliani *et al.*, 1998; Hendzel *et al.*, 2001). Using HSV-1 to suppress host transcription, the studies presented herein examine the spatial organization of HATs and HDACs, assess the levels of transcription-associated histone acetylation, measure the catalytic activity of HATs and HDACs, and study the nature of the viral compartments in terms of constituent host proteins, including HATs, HDACs, associated and unassociated proteins. This has laid the foundation to begin to address the means by which the nucleus becomes reorganized in order to shed light on the nature of nuclear architecture and compartmentalization itself. In this introduction, chromatin structure and function, histone acetyltransferases and deacetylases, spatial organization and dynamics of the nucleus, their implications on transcription, as well as HSV-1 infection are discussed.

1.2 Chromatin Structure and Function

In eukaryotic nuclei, DNA exists in a hierarchical series of nucleoprotein structures called chromatin whose function is to compact and organize the genetic material. The fundamental repeating unit of chromatin is the nucleosome, which consists of approximately 147 base pairs of DNA wrapped 1.65 times in a left-handed turn along the exterior of a core octamer of highly basic, globular proteins called histones (Figure 1.1). The octamer consists of a tetramer of histones H3 and H4 as well as two dimers of H2A and H2B in the linkage (H2A/H2B)-(H4/H3)-(H3/H4)-(H2B/H2A) (Arents *et al.*, 1991). Histones and their linkage pattern with one another in the octamer are highly conserved throughout species. They maintain a characteristic long hydrophobic alpha helix domain bordered by two short hydrophobic helices (Arents *et al.*, 1991; Arents and Moudrianakis, 1993, 1995).

The nature of the interaction of the core histones with themselves and with DNA is dependent upon the histone fold domains. These are the highly structured and conserved regions of these histones, consisting of three alpha helices separated by two loop regions (Arents *et al.*, 1991). It is also dependent upon the interactions with their amino-terminal tails, subject to both reversible and non-reversible covalent post-translational modifications, that protrude from the DNA superhelix (Luger *et al.*, 1997; Luger and Richmond, 1998). The nucleosome is stabilized into a functional unit called the chromatosome with the addition of the linker histone H1 and approximately sixty base pairs called linker DNA (Pruss *et al.*, 1996). In contrast to the core histones, linker histones are not well conserved between species and their function, including their role in chromatin remodeling and transcription, remains ambiguous and contentious (Zlatanova *et al.*, 2000; Kasinsky *et al.*, 2001; Khochbin, 2001). For this reason and for the purposes of this study, hereto, references made to histones will refer to core histones unless otherwise indicated. What is known about core and linker histone interaction is that H1 and the histone termini control the entry and exit of linker DNA into chromatin (An *et al.*, 1998; Zlatanova *et al.*, 1998) and, along with core histone tails, stabilize chromatin (Leuba *et al.*, 1998; Zlatanova *et al.*, 1998).

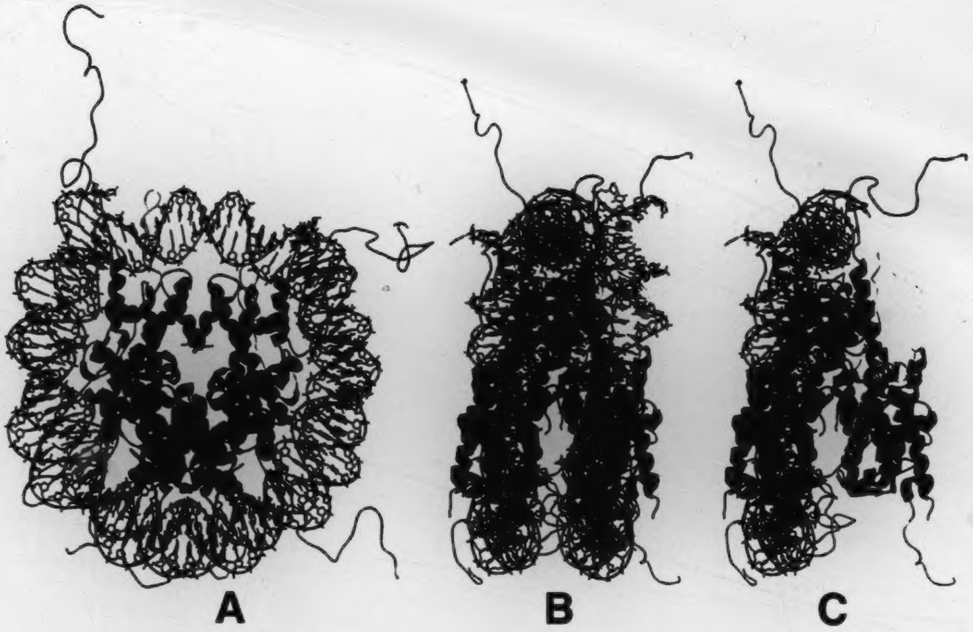

Figure 1.1 Three dimensional rendering of the nucleosome core particle. The nucleosome is composed of 147 base pairs of DNA, shown in black, and 941 amino acids composing the core histones. These are two H2A histones (yellow), two H2B histones (red), two H3 histones (blue) and two H4 histones (green). View A shows the nucleosome through the pseudo-dyad axis. View B shows of the nucleosome down the superhelical axis (view A rotated 90° with respect to the pseudo-dyad or vertical axis), illustrating further the position of the histone tails. View C is the same view as view B with half of the DNA removed to further reveal the position of the core histones. X-ray crystal structure coordinates at 2 Å resolution (Davey *et al.* 2002) were obtained from the RCSB Protein Databank (Berman *et al.* 2000). SwissPdv-Viewer v3.7A and PovRay v3.5 software were used for three dimensional rendering.

The simplest form of chromatin is the linear 10 nm fibre, which may classically be described as "beads on a string". It is well-characterized biochemically (Wolffe, 1998). Rarely if ever does this conformation statically occur in the living cell, if only for the practical space constraints within the nucleus. Nucleosomes are condensed into stable higher-order structures, whether it is short-range folding into the 30 nm fibre or larger associations creating highly condensed interphase and mitotic chromosomes (Figure 1.2). Chromatin structure, once believed to be structurally and operationally inert, is now recognized as a key participant in important cellular processes like transcription, replication and recombination (Fletcher and Hansen, 1996). It is now apparent that chromatin folding is a highly dynamic and regulated process.

Histone amino-terminal tails, so named for their protrusion from the nucleosome core, are the most accessible fraction of the core histone mass and are thus good candidates to mediate or facilitate inter and intranucleosomal interactions. Histone amino-terminal tails are lysine-rich and the net positive charge of these amino acids interacts electrostatically with the net negative charge of the DNA helix deoxyribose-sugar phosphate backbone, favoring a compact conformation of chromatin. While these electrostatic interactions have been used to explain the closed inactive conformation of chromatin and is considered the classic view, there is an increasing body of evidence to support the role of N-terminal tails in protein-protein interactions that are not based in electrostatics (Hansen *et al.*, 1998). As well, basic nucleosome integrity seems largely independent of histone tails. Proteolytic cleavage of N-terminal tails does not seem to significantly affect the typical conformation or assembly of nucleosomes *in vitro*, but does affect the assembly of chromatosomes when depleted of H1. This indicates a role in chromatin structure outside the basic nucleosomal unit (Ausio *et al.*, 1989; Hayes *et al.*, 1991; Garcia-Ramirez *et al.*, 1992). Also, the tails alone are necessary for higher-order ultrastructure self-assembly and compaction (Fletcher and Hansen, 1996; Tse and Hansen, 1997). Therefore, N-terminal histone tail interactions may be correlated with specific fibre conformations, suggesting that particular post-translational modifications may alter specific functional or conformational states in a defined, measurable manner.

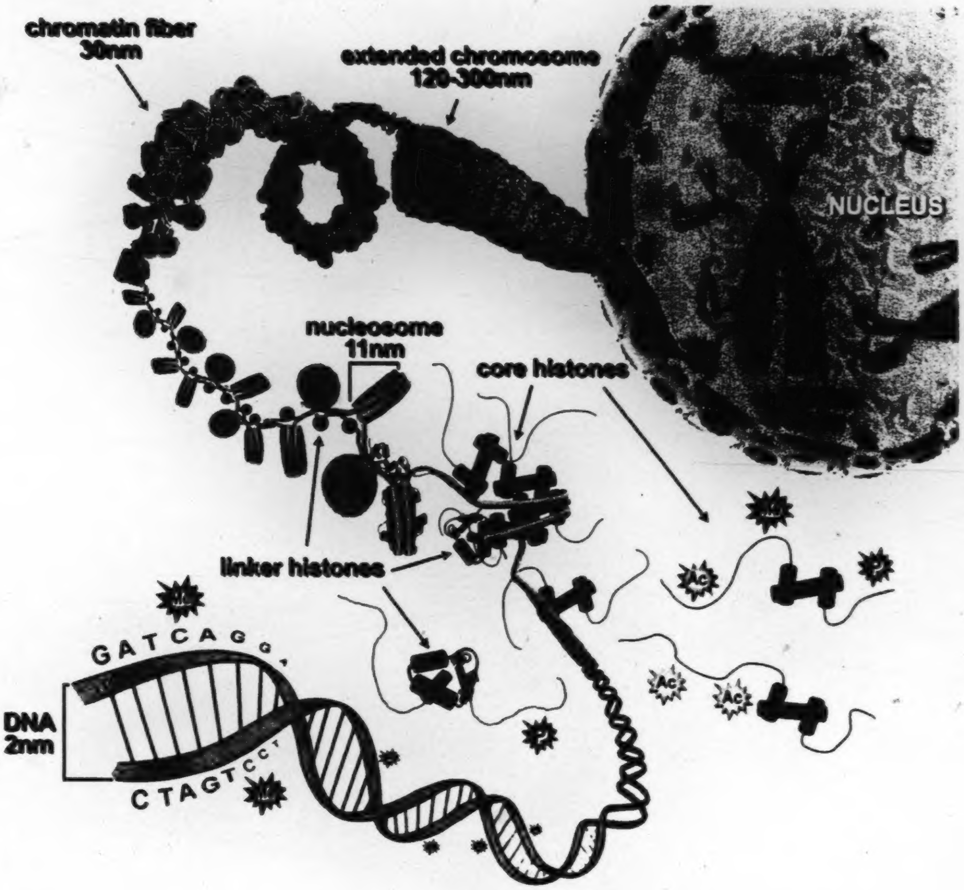
chromatin fiber 30nm
extended chromosome 120-300nm
NUCLEUS
nucleosome 11nm
core histones
linker histones
Ac
Ac
Ac
DNA 2nm
GATCAGG
CTAGTCCT

Figure 1.2 A diagram of chromatin structure. This figure shows the successive levels of chromatin folding, from free DNA to its packaging within the nucleosome to the formation of higher order structures and finally a condensed metaphase chromosome. Included are the approximate measurements of each chromatin conformation. Starbursts on core histones represent post translational modifications of the histone tails. Starbursts on the free DNA fibre represent methylation sites. Artwork modified with permission from the author, Dr. J. Lewis.

There are several known post-translational modifications of core histones, including acetylation, methylation, phosphorylation, ubiquitination, and ADP-ribosylation. The impact of most of these modifications is relatively unexplored and raises unique and differing questions regarding their relevance to nuclear processes (reviewed in Wolffe and Hayes, 1999). In contrast, the structural and functional consequences of core histone acetylation are extensively characterized, primarily due to the early identification of the relationship between transcription and acetylation.

There are two types of histone acetyltransferase activities, A-type and B-type. B-type HAT activities are cytoplasmic and are thought to catalyze acetylation events relating to the transport of newly synthesized histones into the nucleus for incorporation into new nucleosomes (Ruiz-Carrillo *et al.*, 1975; Allis *et al.*, 1985; Roth *et al.*, 2001). A-type acetylation is nuclear and relates to events involved with transcription and thus, this type of acetylation will be the focus for this discussion (Brownell and Allis, 1996).

1.3 Transcriptionally Relevant Chromatin Modification due to Histone Acetylation

The reversible post-translational modification of the core histone N-terminal lysine residues occurs through the enzymatic addition of an acetyl functional group, presumably from an acetyl-CoA donor, to the lysine epsilon-amino group (Figure 1.3). This results in a neutralization of its positive charge and a decreased affinity for DNA (Allfrey, 1966; Hong *et al.*, 1993). Experiments have demonstrated, however, that these electrostatic charge differences only produce minor conformational alterations in the single nucleosome (Libertini *et al.*, 1988; Ausio, 1992; Mutskov *et al.*, 1998). Acetylated core histone tails maintain contact with DNA, suggesting a further and continuous role in

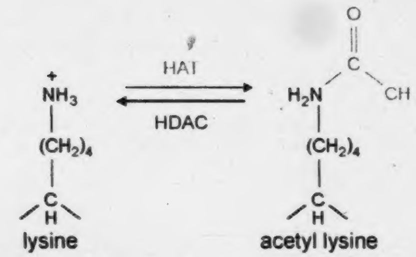

Figure 1.3 Acetylation and deacetylation of the lysine residue.

histone tail interactions through acetylation (Ebralidse *et al.*, 1993; Mutskov *et al.*, 1998). Acetylation, known for many years to protein chemists as a means to alter the secondary structure of proteins, appears to have a major role in altering the conformation of higher-order chromatin structure from the 30 nm fibre and beyond (Baneres *et al.*, 1997; Luger *et al.*, 1997; Hansen *et al.*, 1998; Luger and Richmond, 1998; Tse *et al.*, 1998). In addition, acetylation affects the binding of regulatory proteins to histone tails, suggesting a mechanism whereby the organization of chromatin may be modulated in response to environmental signals (Hecht *et al.*, 1995; Edmondson *et al.*, 1996).

Actively transcribed regions of chromatin maintain the presence of histones (Lacy and Axel, 1975), suggesting that chromatin assumes an accommodating conformation or organization for transcription machinery access. The benchmark for the identification of active versus inactive chromatin was shown to be sensitivity to DNase I digestion (Weintraub and Groudine, 1976), which was the catalyst for many mapping studies of chromatin structure to be undertaken. This technique showed a correlation with both transcriptional states of genes and constitutive sensitivities related to nucleosome-free regions, positioned nucleosomes, DNA-bound non-histone proteins, and other conformationally altered chromatin (reviewed in (Krebs and Peterson, 2000).

Allfrey and others proposed the existence of a relationship between gene expression and the acetylation and methylation of histones (Allfrey *et al.*, 1964). This association with actively-transcribed chromatin was furthered with the discovery of enriched acetylated histones. Later, using chromatin immunoprecipitation (ChIP) assays, specific acetylated histone isoforms correlating with DNase I sensitivity were identified (Sealy and Chalkley, 1978; Vidali *et al.*, 1978; Hebbes *et al.*, 1988). DNase sensitivity and histone hyperacetylation in connection with gene activation itself was reported with the chicken β-globulin locus (Hebbes *et al.*, 1994). Currently, core histone acetylation and general sensitivity to nuclease digestion are now often recognized as indicative properties of transcriptionally active or competent chromatin (Grunstein, 1997; Struhl, 1998).

Recent acetylation studies have focused mainly on genetics and the activity of specific promoters for genes. As an example, acetylation of individual nucleosomes allows the recruitment of isolated individual transcription factors to their recognition elements, while specific site mutagenesis of histones H3 and H4 prevents transcription factor association (Wolffe *et al.*, 1993). New evidence that promoter regions alone are not only acetylated but that regions within and surrounding the gene are acetylated may shift the focus again to the idea that the disruption of heterochromatin structure is the primary necessity for transcriptional activation (Madisen *et al.*, 1998; Hebbes and Allen, 2000).

The threshold of acetylation needed for both significant conformational alteration and elongation to permit transcriptional initiation has been quantified. It is defined as 46% of the maximum site occupancy, or 12 acetyl lysine residues out of a possible 28 (Tse *et al.*, 1998). The ability of transcription factors to access promoter sites on chromatin from which the histone tails are removed is comparable *in vitro* to that on chromatin containing hyperacetylated histones (Lee *et al.*, 1993). This is further evidence to support the notion that the physical disruption of chromatin is the major function of acetylation.

Core histones are amongst the most highly conserved proteins, and the histone lysine residues that undergo acetylation are also highly conserved through species (Roth *et al.*, 2001; Figure 1.4). Acetylation site specificity, order, and lysine residue preference add complexity to acetylation that allows functionally specific alterations and sensitive control over histone interactions and chromatin ultrastructure. When pig thymus and HeLa cells were treated with the reversible histone deacetylase inhibitor butyrate, it was observed that histone H2B has preferential mono- and di-acetylations on lysines 12 and 15 over lysines 5 and 20. For histone H4, lysine 20 is always acetylated or methylated and lysine 16 is exclusively di-acetylated. Additional acetylation preferences generally extend from this site to the N-terminus from lysine 12, to lysine 8 and lysine 5, the final site being primarily exclusive to the penta-acetyl form. There also seems to be a rigid order of site occupancy for H3. The acetylation preference begins at lysine 14, then lysine 23, then lysine 18 and finally lysine 9 (Thorne *et al.*, 1990). There is only one possible acetylation site for H2A.

The existence of this hierarchy of acetylation sites, and indeed the amount of variation that is shown to exist between the histone tails, reflect functionally distinct roles. The generalizations of simple hyperacetylated or hypoacetylated chromatin controlling gene activation or repression do not hold true when specific acetylated histone species are studied, as is demonstrated with specific acetylated H4 isoforms. It is the specific pattern of lysine acetylation rather than the bulk acetylation level that may be of

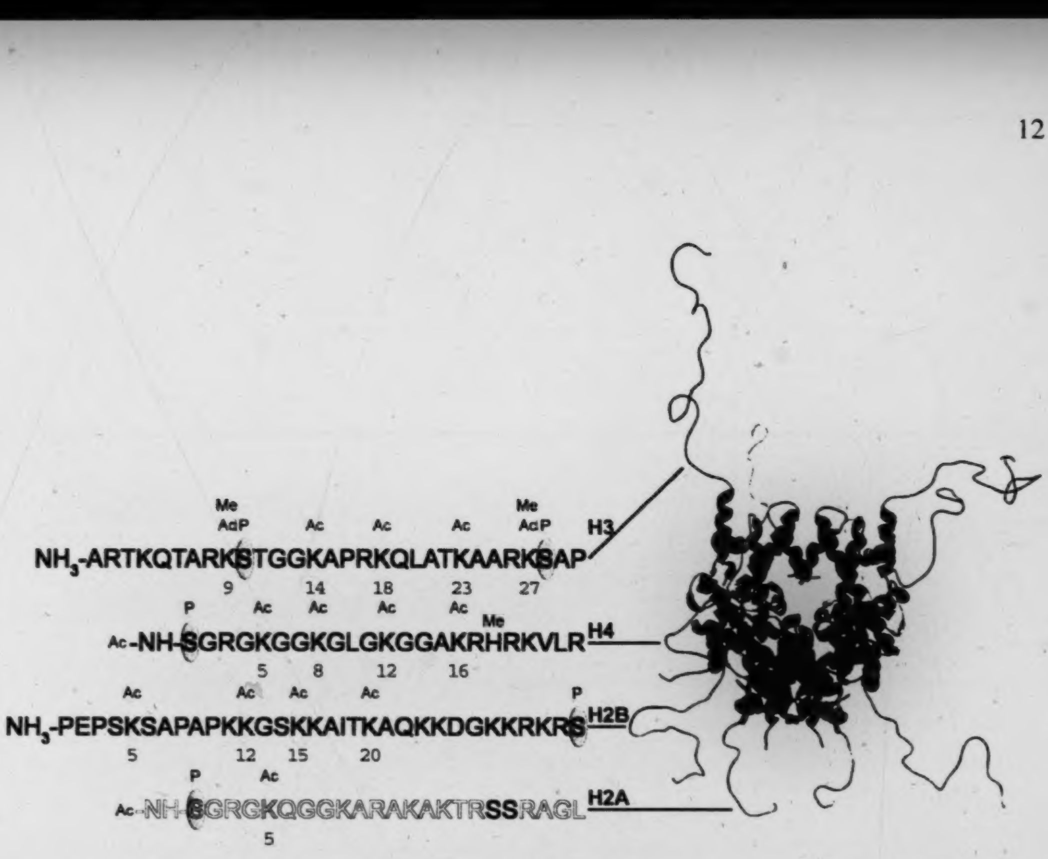
Me
AcP
Ac
Ac
Ac
Me
AcP
H3
NH3-ARTKQTARKSTGGKAPRKQLATKAARKSAP
9
14
18
23
27
P
Ac
Ac
Ac
Ac
Me
H4
Ac-NH-SGRGKGGKGLGKGGAKRHRKVLR
5
8
12
16
Ac
Ac
Ac
Ac
P
H2B
NH3-PEPSKSAPAPKKGSKKAITKAQKKDGKKRKRS
5
12
15
20
P
Ac
H2A
Ac-NH-SGRGKQGGKARAKAKTRSSRAGL
5

Figure 1.4 Sites of post-translational modifications on the amino terminal tails of human core histones. The amino acid sequence of the N-terminal tails is shown to the left of the histone core particle and color coded to match the corresponding histone. The modifications shown are known acetylation (Ac), phosphorylation (P) and methylation (Me) sites. Numbers indicate the position of the lysine residue above. Light blue serine and histidine residues represent potential phosphorylation sites. Three dimensional rendering of the nucleosome (right) was performed as in Figure 1.1. Based on figures by Spencer and Davie, 1999 and Cheung *et al.*, 2000.

functional importance (Turner *et al.*, 1992; Johnson *et al.*, 1998; Rundlett *et al.*, 1998). Acetylation of H4 appears to play a major role in chromatin structural changes that mediate enhanced binding of transcription factors to their recognition sites within nucleosomes (Vettese-Dadey *et al.*, 1996). H2B has little relevance to chromatin remodeling and its role in permitting transcription is ambiguous. Acetylated histone H2B is associated with transcriptionally permissive chromatin (Hendzel and Davie, 1990; Puerta *et al.*, 1995). However, this acetylation has shown not to be responsible for sufficient alteration of nucleosome structure, but rather stabilization of tetrameric H3-H4 hyperacetylated species (Morales and Richard-Foy, 2000). A new model for acetylated H2A-H2B has been hypothesized outlining the loss of these acetylated dimers to a chaperone molecule NAP-1 during remodeling by acetylation. This is as a result of an initial ATP-dependent chromatin remodeling event. This loss of the H2A-H2B dimer theoretically allows for HAT dependant chromatin remodeling of the nucleosome and facilitated transcription. While the theory is controversial, the results bolsters the increasingly popular view that H3 and H4 acetylation are more predominant players in transcriptional control, partially due to the abundance of A-type acetylation events, with preferences to tetrameric lysine residues (Ito *et al.*, 2000; Roth *et al.*, 2001). There are numerous accounts of the significance of H3 hyperacetylation in transcriptionally facilitative chromatin remodeling and factor recruitment (Hebbes *et al.*, 1988; Boggs *et al.*, 1996; Hendzel *et al.*, 1998). Most significant is recent evidence for a multi-faceted model of gene activation, discussed more thoroughly in later chapters, that dissociates transcriptional activation from chromatin remodeling as having distinct mechanisms. Promoter activation simply requires the hyperacetylation of H3 and occurs subsequent to chromatin disruption (Schübeler *et al.*, 2000). Once these events have occurred, specific acetylated species may orchestrate the recruitment of additional specific factors necessary for transcription.

It is important to establish that at any time during a non-replicative stage in the cell cycle, chromatin is only acetylated to the mono- and di-acetylated forms, with the exception of approximately 5% of chromatin that achieves transcriptionally relevant hyperacetylated states. Histone acetylation is a dynamic process occurring at two different rates, as does the subsequent deacetylation, and therefore these two differing states have distinct classifications. When mammalian cells are treated with the reversible histone deacetylase inhibitor sodium butyrate, the majority population of core histones is acetylated ($t_{1/2}$=200–300 min for monoacetylated H4) and deacetylated at a slower rate ($t_{1/2}$=30 min; Davie, 1997; Spencer and Davie, 1999). This is considered class II histone acetylation, or bulk acetylation. Class I dynamic histone acetylation is characterized by the ability of H3 and H4 populations to rapidly hyperacetylate and subsequently quickly deacetylate with the treatment and removal of inhibitor respectively (Hendzel *et al.*, 1991). This small population of core histones is characterized by rapid hyperacetylation ($t_{1/2}$=7 min for monoacetylated histone H4) and rapid deacetylation ($t_{1/2}$=3–7 min). The second Class I histone acetylation is considered to be associated with transcription as these hyperacetylated histones are principally bound to active DNA (Ip *et al.*, 1988; Boffa *et al.*, 1990; Hendzel *et al.*, 1991) while class II histones are found in repressed chromatin, although within domains of gene-enriched chromatin fragments (Hendzel and Davie, 1991). An important goal in studying this specific acetylation phenomenon has been the isolation of native activities and factors that generate such specific patterns of acetylation.

1.4 Histone Acetyltransferases and Deacetylases

Although it was well established that histone acetylation correlated with transcriptionally active competent chromatin, decades passed before evidence for core histone acetylation as a prerequisite step for transcription was produced (Clayton *et al.*, 1993). The identification of specific enzymes with histone acetyltransferase activity provides a direct link to gene transcription. The first histone acetyltransferase (HAT) to be cloned was *Tetrahymena* nuclear HAT p55. This HAT had considerable homology to the yeast transcription factor Gcn5 and thus helped strengthen the direct correlation between histone acetylation and transcriptional regulation (Brownell *et al.*, 1996; Kuo *et al.*, 1996). Yeast Gcn5 (yGcn5) alone has the ability to acetylate free H3 but is not able to interact with the nucleosome and must be a member of a multiprotein complex in order to acetylate these histones (Utley *et al.*, 1998 but see Tse *et al.*, 1998; Sendra *et al.*, 2000). Gcn5 is recruited to DNA sequence-specific promoters through Ada proteins and this complex also interacts with components of the basal transcription machinery, the SAGA (Spt-Ada-Gcn5-acetyltransferase) complex (Grant *et al.*, 1998). The recruitment of the SAGA complex by transcriptional activators leads to localized nucleosome acetylation *in vitro* and *in situ* and, notably, transcription is dependent upon this HAT activity (Davie and Chadee, 1998; Grant *et al.*, 1998; Kuo *et al.*, 1998). These multipartite, multifaceted aggregations, which are important to the facilitation of transcription through chromatin remodeling, recruitment of factors and basal transcription itself, lead to the new classification of these complexes as transcriptional coactivators (reviews in (Brown *et al.*, 2000; Lemon and Tjian, 2000). Significant overlap of factors or factor aggregations, including HATs, contained within coactivator complexes, indicates that coactivators are modular in nature and dynamic to respond to the continuously changing needs of the cell (Näär *et al.*, 2001). This also proves to be a general model for HAT recruitment to specific promoters and initiated the search for and identification of other coactivator proteins with intrinsic HAT activities.

While numerous HATs continue to be sought after and identified, the common families represented within this study are introduced here. Yeast Gcn5 has been the most studied and remains the paradigm for HAT work *in vitro* and *in vivo*. Those HATs with related structural motifs to yGcn5 are placed in the GNAT (Gcn5-related *N*-

acetyltransferase) superfamily. The human GCN5 is isolated (Candau *et al.*, 1996; Wang *et al.*, 1997) as is its nuclear ortholog p300/CBP associated factor (P/CAF or PCAF; (Yang *et al.*, 1996b) and their complexes are considered to be the human counterparts of the yeast SAGA. As its name suggests, PCAF was found to be an associated factor of other coactivators, including cyclic-AMP response element (CREB) binding protein (CBP) and p300 as well as the nuclear receptor coactivator ACTR, all of which are acetyltransferases (Bannister and Kouzarides, 1996; Ogryzko *et al.*, 1996; Chen *et al.*, 1997).

CBP and p300 are perhaps two of the most studied transcriptional coactivators as they are capable of acetylating all core histones and implicated in processes including cell growth, development and oncogenesis. They are generally grouped together as sequential and functional homologues, although important distinctions are currently being revealed (Ugai *et al.*, 1999; Yuan *et al.*, 1999). As they are present in limited amounts within cells, often there is competition between other proteins to recruit and interact with these proteins either directly or indirectly, and this possibly provides a mechanism for functional selection, regulation and integration. CBP/p300 regulates essentially all known pathways of gene expression and the transcriptional coactivator properties of CBP are utilized by at least twenty-five different transcription factors representing nearly all known classes of DNA binding proteins (Goodman and Smolik, 2000; McManus and Hendzel, 2001; Näär *et al.*, 2001). Factors that interact with CBP/p300 include nuclear hormone receptors (Kamei *et al.*, 1996; Kino *et al.*, 1999), phosphorylated CREB as a response to the cAMP signaling pathway (Kwok *et al.*, 1994) as well as c-fos/c-jun transcription factors and proto-oncogenes activated by RAS/MAPK-mediated phosphorylation (Bannister *et al.*, 1995; Janknecht *et al.*, 1995). It is also a member of the RNA polymerase II holoenzyme and interacts directly with TATA box-binding protein (TBP; also known as transcription factor IID; TFIID) and transcription factor IIB (TFIIB). TFIIB binds TBP and combined are the nucleating factors that recruit RNAPII to the initiation complex. Transcriptional initiation is integral to the transcription process and successful transcriptional activation hinges on this rate-limiting step. Therefore, CBP/p300 is intimately involved in this process at the most critical stages and its activity modulates chromatin ultrastructure necessary for transcription.

There are also other key families of enzymes with A-type HAT activity. ACTR (also called steroid receptor coactivator-3, SRC-3), along with its HAT counterpart SRC-1, are referred to as nuclear receptor cofactors and bind a variety of nuclear receptors in a ligand-dependent manner. Ligands activate nuclear receptors and have the potential to recruit multiple coactivators that share HAT activities (Davie, 1998; Torchia *et al.*, 1998). TAFII250 (TATA-binding protein-associated factor), classified as a basal transcription factor and part of the TBP (TFIID) complex, is also proven to have Gcn5-like histone acetyltransferase activity *in vitro* (Mizzen *et al.*, 1996; Wassarman and Sauer, 2001). The final nuclear acetyltransferase family is MYST, named for its founding members MOZ, Ybf2/Sas3, Sas2 and Tip60 identified primarily in yeast. This family is defined by the presence of a common acetyl-CoA binding structural motif, which is thought to be the catalytic substrate for acetylation. HAT activity has yet to be identified in all members of the MYST family (Roth *et al.*, 2001; Pelletier *et al.*, 2003).

Until recently, HAT activity was believed to be limited to lysine moieties on histone substrates. Factor acetyltransferase activity (FAT) is a posttranslational modification of lysine residues on other non-histone target proteins and it is also implicated in transcriptional activation of specific genes. FAT activity has been identified in PCAF and CBP/p300. Acetylation of p53 tumor suppressor protein by CBP is shown to increase the substrate's affinity for sequence-specific DNA (Gu *et al.*, 1997) and acetylation of ACTR by CBP occurs as a result of hormone-induced estrogen receptor related gene activation (Chen *et al.*, 1999). TAFII250, PCAF and p300 are able to acetylate members TFIIE and F of the TBP transcription complex with undetermined functional consequences (Imhof and Wolffe, 1998). While the stimuli for and consequences of FAT have yet to be elucidated, the substrates are associated with other multifunctional proteins to form coactivator complexes. Acetylation may be a signaling or activation mechanism necessary for the coordination of transcriptional initiation.

In the same year that histone acetyltransferases were identified, enzymes displaying the reverse catalytic activity, histone deacetylases (HDACs), were discovered. The first one to be described, HDAC1, is related to the transcriptional regulator in yeast RPD3 (Taunton *et al.*, 1996). Almost concurrently, another HDAC was discovered (HDAC2) with homology to RPD3 and found to interact with the ubiquitous transcriptional coactivator and corepressor YY1 (Yang *et al.*, 1996a). Not only was histone deacetylation known to repress transcription by imposing a closed chromatin conformation, but these discoveries established an early primary link between histone deacetylases and transcription modifying proteins (Wolffe and Guschin, 2000). It was also direct evidence for the well-established correlation that histone deacetylation was linked with transcriptional repression.

Histone deacetylases are grouped into three families: Classes I, II and III. Class I

HDACs are known as the RPD3-like histone deacetylase family, due to their relatedness to the *Saccharomyces cerevisiae* transcriptional regulator RPD3, and include the members HDAC1, HDAC2, HDAC3 and HDAC8. These proteins grouped according to the homology of their catalytic sites. HDAC1 and 2 are highly homologous with 85% identical amino acid sequence and 75% identical gene sequence in humans and average 82% homology through species (Cress and Seto, 2000; de Ruijter *et al.*, 2003). All class I HDACs are expressed in most tissues and cell lines and are primarily nuclear proteins (de Ruijter *et al.*, 2003). As they lack a nuclear export signal, HDAC1 and HDAC 2 localize exclusively to the nucleus where they accumulate in discrete foci (Emiliani *et al.*, 1998; Hendzel *et al.*, 1998; Johnstone, 2002). HDAC3 contains both a nuclear import and export signal but remains primarily nuclear, perhaps in part due to an abundance of binding sites and associations with corepressor complexes and other HDACs (Bertos *et al.*, 2001; Fischle *et al.*, 2001; Fischle *et al.*, 2002; Yang *et al.*, 2002). HDAC1 and HDAC2 are responsible for the majority of deacetylase activity in cells (de Ruijter *et al.*, 2003). HDAC1, the most studied HDAC to date, has had its interactions with other factors determined primarily through the study of its interacting factors rather than HDAC1-directed research, suggesting a wide-spread role in transcriptional repression, cell cycle regulation, oncogenesis, and possibly DNA replication and repair (Cai *et al.*, 2000; Cress and Seto, 2000; Wade, 2001; Milutinovic *et al.*, 2002; Fernandez-Capetillo and Nussenzweig, 2004). These interactions are evidence suggesting that HDAC1, and to a lesser extent HDAC2, cannot exert activity *in vivo* without the formation of complexes with transcriptional repressors, cell cycle checkpoint molecules, and chromatin modifying enzymes (Spencer and Davie, 1999; Burke and Baniahmad, 2000;

Cress and Seto, 2000).

Transcriptional corepressors have intrinsic silencing ability and actively inhibit transcription. Although they are essential for a wide range of biological pathways and homeostatic mechanisms, including differentiation, development, cell proliferation, and apoptosis, they lack the ability to directly bind DNA. They are recruited by factors bound to the regulatory regions of target genes where they contribute to the silencing ability of transcriptional silencers or repress the transcriptional activity of gene activators (Burke and Baniahmad, 2000). Perhaps the most studied and characterized mechanism of corepressor function is through the recruitment of enzymes with HDAC activity. Corepressor-HDAC complexes contribute to gene repression through either specific targeting of deacetylase activity by repressors or non-targeted deacetylation through constitutive association with chromatin (Li *et al.*, 2002).

HDACs 1 and 2 are known to be members of three distinct and well-characterized corepressor complexes, the SIN3 complexes, the nucleosome remodeling and deacetylase (NuRD) complex, and the CoREST/kiaa0071 complex (Heinzel *et al.*, 1997; Zhang *et al.*, 1998; You *et al.*, 2001). SIN3 proteins bind to various silencers and recruit HDAC activities. These rudimentary complexes in turn are able to form incrementally larger complexes through recruitment of nuclear hormone receptors that are ligand-regulated transcription factors. Hormone-sensitive interaction with certain corepressor proteins such as nuclear receptor co-repressor (NCoR) and its related protein, silencing mediator for retinoic acid and thyroid hormone receptor (SMRT), negatively affect transcriptional activities (Alland *et al.*, 1997; Hassig *et al.*, 1997; Heinzel *et al.*, 1997). Covalent histone modifications often work in concert, as is the case with methylation and deacetylation, in the formation of repressive chromatin structures and gene silencing (Jones *et al.*, 1998). This occurs when SMRT and mSin3A methyl-CpG-binding protein 2, binds to Sin3. This causes the recruitment of an HDAC1/HDAC2 separate composite complex (known as Mi-2) and thus facilitates repression.

Class II histone deacetylases, including HDAC4, HDAC5, HDAC6, HDAC7, HDAC9 and HDAC10, all contain a domain homologous to the deacetylase domain of HAD-1 in yeast. HDAC4, 5, 7, and 10, like their class I counterparts, function as transcriptional corepressors and are able to deacetylate acetyl lysine groups, form large multiprotein complexes and play a role in differentiation and proliferation (Wang *et al.*, 1997; Bertos *et al.*, 2001; Fischle *et al.*, 2001; Tong *et al.*, 2002). Sensitivity to histone deacetylase inhibitors has been demonstrated for HDACs 4, 5 and 6 (Fischle *et al.*, 1999; Grozinger *et al.*, 1999; Wang *et al.*, 1999). Distribution of HDAC4, 5 and 7 shows primarily nuclear and distinct subnuclear localization as well as a smaller cytoplasmic population (Fischle *et al.*, 1999; Wang *et al.*, 1999; Grozinger and Schreiber, 2000; Fischle *et al.*, 2001) while HDAC6 is primarily cytoplasmic (Bertos *et al.*, 2001; Gao *et al.*, 2002). Subcellular compartmentalization is achieved and maintained through site-specific phosphorylation and binding the 14-3-3 protein. This protein has been shown to export HDAC4 and HDAC5 from the nucleus and override its strong intrinsic nuclear localization signal by remaining bound in the cytoplasm (Grozinger and Schreiber, 2000; Wang *et al.*, 2000; Wang and Yang, 2001). HDAC 9 and HDAC10 have both nuclear populations, dependent upon the splice variant, and cytoplasmic populations maintained by associations with unknown factors (Bertos *et al.*, 2001; de Ruijter *et al.*, 2003). While the functions and associations for class II HDACs are far from being completely elucidated, certain members are known to be able to form multiprotein complexes. Certain reports indicate that human class II enzymes, specifically HDAC4 and HDAC5, are able to form a complex with the hormone receptor corepressors NCoR and SMRT, along with the class I HDAC3, but without the mSin3A adaptor protein (Huang *et al.*, 2000; Kao *et al.*, 2000). In the case of HDAC4, this interaction regulates transcription by bridging the NCoR/SMRT-HDAC3 complex in the absence of any intrinsic class II HDAC activity (Fischle *et al.*, 2002).

Class III histone deacetylases, also called the NAD+ dependent Sir2-like histone deacetylases, have recently been discovered but as they are cytoplasmic HDACs (Afshar and Murnane, 1999), they will not be discussed. HDAC 11 has recently been identified as a nuclear HDAC (Gao *et al.*, 2002) but its class has yet to be determined and, consequently, it will also be excluded from this review.

1.5 Histone Acetylation and the Process of Transcriptional Activation

Chromatin occupies approximately fifteen percent of the nuclear space in distinct zones termed chromosome territories as shown by *in situ* hybridization studies (Lamond and Earnshaw, 1998; Zink *et al.*, 1998; Munkel *et al.*, 1999; Cremer *et al.*, 2000). This modular and dynamic chromosome territory organization can be subclassified by its higher order chromatin organization. These morphological classifications in interphase cells are referred to as either "open" euchromatin or "closed" heterochromatin. The morphology is both a consequence of its transcriptional state and concomitantly its position relative to the dynamic enrichment of functionally specific chromatin-related factors in the interchromatin compartment. These may include transcriptional coactivators and corepressors possessing HAT and HDAC activities respectively or proteins that maintain heterochromatic domains (Cremer *et al.*, 2000; Cheutin *et al.*, 2003; Fischle *et al.*, 2003b).

As mentioned, interplays between the net activities of HATs and HDACs dictates the steady-state level of acetylation at a chromatin site. Although HDACs are implicated in transcriptional repression, perhaps counterintuitively, they primarily function at sites within the genome that are highly acetylated (Vogelauer *et al.*, 2000; Katan-Khaykovich and Struhl, 2002). Consequently, HDACs preferentially act on the same subset of chromatin, the transcriptionally competent and active chromatin, as do the HATs. Recently it has been shown that HATs and HDACs exist in close spatial proximity within the nucleus *in vivo*, confirming their local antagonistic enzymatic activities on the same subset of chromatin (Yamagoe *et al.*, 2003). However, how distinctive, individual heterochromatic and euchromatic regions are differentially created and maintained in active and inactive chromatin territories is a question that remains to be completely elucidated.

In understanding the higher-order organization of the interphase genome, structural studies of chromosomes are being reconciled with analysis of transcriptionally active (or repressed) gene loci in the context of their spatial localization within the nucleus. Interphase chromosomes occupy discrete territories and, in some tissue-specific cases, interphase chromosome domains have been reported as having preferred nuclear positions (Manuelidis and Borden, 1988; Nagele *et al.*, 1999). Regions of transcriptional competence in the interphase nucleus are termed permissive chromatin domains (Eberharter and Becker, 2002). Permissive chromatin territories are achieved in discrete areas separate from repressive heterochromatic structures that are localized primarily at the periphery of the nucleus close to the nuclear membrane (Cremer and Cremer, 2001). Gene rich interphase chromosomes have a mass that is more interior in its localization in the nucleus as compared to chromosomes that are gene poor (Croft *et al.*, 1999). Indeed, active gene loci may be the consequence of the non-random organization of chromosomes rather than a property of individual genes as determined by the higher-order spatial organization of the genome (Roix *et al.*, 2003). This is further supported by comparative chromosome territory studies in mammals and non-mammalian vertebrates whereby the spatial relationships of chromosome territories (or segments thereof) to each other and their radial distribution within the nucleus were found to be homologous within the same tissue type across related species and this higher order chromatin arrangement was also correlated with gene density, size and replication timing (Tanabe *et al.*, 2002).

The non-random organization of chromosome territories does not imply that chromatin is a largely static, inactive compartment and that movements are strictly the result of chromatin condensation and decondensation in gene rich and poor chromosome regions respectively. Motion has been seen in human and *Drosophila* chromosomes in localized distances over short periods of time and over longer distances over longer time scales, as well as differential amplitudes of movements dependent upon nuclear positioning and cell cycle, in an ATP dependent manner (Belmont, 2003). The movement of chromatin and the maintenance of chromatin territories have yet to be completely explained. The dynamic nature of other factors in the nucleus will be expanded upon in later chapters.

Recent evidence suggests that broad euchromatin domain-wide acetylation patterns exist. These are reported for some housekeeping and cell type specific genes, rather than strictly on specific active promoter nucleosomes and they may be correlated to class II global histone acetylation. This state, characterized by intermediate levels of H3 and H4 acetylation, may be brought about by a combination of HAT and HDAC activities that are not specifically targeted to this chromatin area (Vogelauer *et al.*, 2000). A well-characterized examples of this phenomenon is the β-globin locus, which reveals broad acetylation throughout well-defined domains in the interior of the nucleus (Sch*ü*beler *et al.*, 2000; Litt *et al.*, 2001). Consistent with this is the localization of H3 acetylation in the nucleus – depleted on the periphery of the nucleus and enriched in the interior (Hendzel *et al.*, 1998; Hendzel *et al.*, 2001). The acetylation of the β-globin locus is contingent upon its location within the nucleus. However, having an open morphology in the interior of the nucleus, away from centromeric chromatin, it is not necessarily indicative of transcription (Sch*ü*beler *et al.*, 2000).

Acetylation of the histones in permissive chromatin counteracts the tendency for nucleosomal structures to compact, making the chromatin more accessible to interacting proteins. The open morphology of the chromatin fibre is also surprisingly compact, although still less compact than heterochromatin, and measures generally between 100-200nm (Cmarko *et al.*, 1999). This high packing density of genes within nuclear space produced by chromatin folding could provide a basis for the spatial organization of the genome and transcriptional control (Hendzel *et al.*, 2001). The untargeted nature of this acetylation may be necessary for chromatin remodeling that is associated with transcription rather than transcriptional activation itself and the maintenance of this organization.

Domain-wide class II acetylation, introduced in Section 1.3, has properties and functionalities distinct from those of class I histone acetylation. While the acetylation of lysine is chemically and enzymatically identical, class I acetylation is localized, specifically targeted and required for transcriptional activation. It is characterized by the rapid hyperacetylation of histones to the highest state, the tetraacetylated forms of H3 and H4, which can be induced by using the reversible histone deacetylase inhibitor butyrate. Once the inhibitor is removed, the histones are rapidly deacetylated. This is another marked difference between class I and II acetylation – the latter having a much slower rate of deacetylation even with the same acetylation rate (Hendzel *et al.*, 1991). This type of acetylation only persists in a small fraction of the histone population (Hendzel and Davie, 1991). These histones are primarily contained within promoter and enhancer elements. However, they can also be found enriched at boundary or insulator elements of chromosome domains, DNA sequences that have the function of establishing and delimiting domains of expression, along with other DNAse I hypersensitive sites (Litt *et al.*, 2001; Eberharter and Becker, 2002).

It is suggested that class I and class II acetylation may act in tandem to modify chromatin structure and facilitate transcription. Class II histone acetylation may function to accommodate, spatially, substrate-specific coactivator complexes (Brown *et al.*, 2000; Lemon and Tjian, 2000). It is suggested that transcriptional regulation must involve targeting of these enzymes to specific sites. Localized perturbations of this equilibrium can occur by promoter-specific targeting of HATs and HDAC, and rapid recovery rates are indicative of the greater efficiency found for deacetylase activity (Katan-Khaykovich and Struhl, 2002). Well-characterized acetylation preferences and patterns have long been recognized as being important for chromatin modifications (Thorne *et al.*, 1990). In one specific case, acetylated chromatin species lysine 9 and lysine 14 of H3 are required for the recruitment of TATA Box Binding Protein (TBP; TFIID) while acetylation at lysine 8 of histone H4 mediates the recruitment of the SWI/SNF complex (Agalioti *et al.*, 2002). This chromatin remodeling may allow for further targeted histone acetylation and transcriptional initiation (Featherstone, 2002). Taken together, the pattern of non-targeted acetylation in concert with other histone tail modifications, are thought to create complex-specific binding sites and dictate functionality at a particular promoter. Interactions between the acetyl lysine and the molecule are facilitated through bromodomains contained within the chromatin remodeling acetyltransferases (Brown *et al.*, 2000; Strahl and Allis, 2000; Fischle *et al.*, 2003a). There is strong evidence implicating the combination of post-translational modification of histone tails in the long-term maintenance and heritability of transcriptional states as well as transcriptional initiation (Turner, 2002).

It becomes clear in this multi-faceted model of gene activation that chromatin remodeling and transcriptional activation, as a result of different acetylations, have distinct mechanisms. Chromatin remodeling is clearly the prerequisite condition. Yet, both of these complex processes require the coordinated participation of a large number of highly specialized components that must occupy a particular position in order to activate a specific gene synchronously with other factors and cellular processes. Local, targeted acetylation can be explained by HAT activities directed toward specific DNA binding sites via activators. The assembly of the constituent components of these holoenzymes, contingent upon its specific function, is not completely understood. How non-targeted acetylation over large domains, and even the selection and maintenance of permissive chromatin are achieved, is not elucidated. It has been suggested that these entire phenomena are related to the nature of the protein environment, that is, the proximity of the chromatin to and the relationship with a functionally organized interchromatin space.

1.6 Nuclear Compartments

Aside from the previously described organization of the chromatin domain, the interphase interchromatin space is also compartmentalized. Many nuclear factors, including HATs and HDACs, either localize partially or completely into distinct punctate foci within the interchromatin space. As HATs and HDACs act in dynamic equilibrium, the formation of distinctive foci may be explained by a functionally significant organization of these factors. It is useful at this point in the discussion to introduce the concepts of nuclear compartmentalization, how compartments are formed and their dynamic nature. This will allow a more clear understanding of the emerging research into the regulation of histone acetylation as a function of nuclear compartmentalization. This section will review nuclear protein dynamics, illustrated by an overview of compartments that are morphologically well characterized. This will outline how and for what purpose the subnuclear distribution of regulatory proteins might be controlled, and will introduce the current thoughts nature of transcription compartments, including those with acetyltransferase and deacetylases activity.

Compared to cytoplasmic organelles, nuclear compartments are enriched in certain resident proteins within a defined volume and maintain a characteristic morphology and number, but they differ in that they are not enclosed by a membrane. The nuclear bodies range in sizes from tenths of a micrometer to several micrometers. Some of the morphologically distinct and well-characterized nuclear compartments include nucleoli (Carmo-Fonseca *et al.*, 2000) interchromatin granule clusters (IGCs), which are also known as nuclear speckles or more recently termed splicing factor compartments (SFCs; Spector *et al.*, 1991; Hendzel *et al.*, 1998), promyelocytic leukemia (PML) bodies (also called ND10, POD, and Kr bodies; (Ascoli and Maul, 1991; Zhong *et al.*, 2000), the coiled or Cajal Bodies (CBs; Gall *et al.*, 1999), Gemini of coiled body (GEM; Liu and Dreyfuss, 1996), heat shock induced hnRNP containing HAP body (Chiodi *et al.*, 2000), matrix-associated deacetylase body (Downes *et al.*, 2000) and recently the paraspeckles (Fox *et al.*, 2002). The functions of most nuclear compartments remain to be determined. The exception is the nucleolus, which serves as a factory for the transcription and processing of ribosomal RNA and the formation of ribosomes (Lamond and Earnshaw, 1998). Cajal bodies and GEMs, which can colocalize (Liu and Dreyfuss, 1996), and the newly characterized TBP bodies (Wang *et al.*, 2002), which may function as an intermediary compartment between many well-characterized compartments, are the only nuclear compartments that show a direct association among the different nuclear bodies. Compartments, aside from their function or distinct components, possess a number of common general characteristics.

Nuclear compartments are highly dynamic in nature. Most, including the nucleolus, speckles and Cajal bodies, have high structural plasticity. They have been shown to disassemble during mitotic division and reassemble shortly after in the newly formed daughter cells (Spector *et al.*, 1991; Carmo-Fonseca *et al.*, 1993; Dundr *et al.*, 2000). Although compartments often maintain considerable positional stability, changes in shape are observed over time indicating the existence of internal dynamics (Misteli and Spector, 1997; Kruhlak *et al.*, 2000). In the case of splicing factor compartments, reorganization of the compartments into round, larger structures is observed with the inhibition of transcription (Hendzel and Bazett-Jones, 1995). It has also been demonstrated that speckles in transcriptionally active cells have a twofold increase in speckle dynamics over transcriptionally inactive cells (Eils *et al.*, 2000).

Active study of the dynamic nature of proteins *in vivo* identified within compartments has been greatly facilitated by the ability to express green fluorescent protein (GFP)-tagged gene products. Many GFP-tagged nuclear proteins and transcription factors seem to function with little impairment to their normal behavior (Hager *et al.*, 2002). Photobleaching, an extremely useful application that exploits the properties of the GFP fluorophore, has allowed protein movement to be much more precisely observed and quantitated (Misteli and Spector, 1997). Particularly useful are Fluorescence Recovery after Photobleaching (FRAP) experiments, in which a small area of a cell expressing a GFP fusion protein is rapidly and irreversibly bleached using a targeted laser pulse. Bleaching generates a region devoid of the fluorescence signal. The recovery of the fluorescence signal is due to the influx of unbleached molecules into the bleached area, and is measured as a function of time using time-lapse microscopy. Fluorescence Loss in Photobleaching (FLIP) is similar to FRAP in that a region of fluorescence within the cell is repeatedly bleached but the area outside the region is monitored for a loss in fluorescence. The kinetics of recovery or depletion contains information about the apparent mobility of the labeled proteins, usually on the order of seconds for proteins that are not freely diffusible (Kruhlak *et al.*, 2000; Dundr and Misteli, 2001).

It has been shown using FRAP that there is a continuous flux of factors exchanging between the nucleoplasm and the compartment while, as a whole, the compartment maintains integrity (Huang *et al.*, 1998; Kruhlak *et al.*, 2000; Phair and Misteli, 2000; Snaar *et al.*, 2000). What is observed as a morphologically distinct and stable structure in the nucleus, a nuclear compartment, is actually factors constantly exchanging. This contributes to changes in its shape and composition over time. This, as previously mentioned, may be due to the functional status of the compartment and the proteins from which it is comprised. Recent mathematical modeling using FRAP data will allow further studies into the distribution between different populations. Determination of rates of diffusion and association and dissociation constants is possible by this method (Carrero *et al.*, 2003; Gerlich *et al.*, 2003).

The characteristics of nuclear compartments outlined previously leads to another important aspect, the notion that self-organization may be a means of compartment formation and maintenance (Misteli, 2000). Using an example of a functionally characterized compartment, it is observed that nucleolus formation is ribosomal RNA transcription-dependent (Lamond and Earnshaw, 1998; Olson *et al.*, 2000). Generally, protein self-interaction seems to be a feature of nuclear compartments (Hebert and Matera, 2000). This could serve, in part, as an explanation for the formation of nuclear foci as a means of concentrating factors for functional reasons or for sequestration of factors away from sites where their activity is not needed or detrimental to a particular function (Misteli, 2000).

1.7 Nuclear Protein Dynamics and Compartment Formation

The debate concerning the structure and functional organization of the nucleus ranges between the extreme views that the nucleus is a highly rigid organelle with a nucleoskeleton and its own distinct organelles to a theory where the nuclear membrane is simply a barrier that holds the nucleic acid and protein factors that move freely within a gelatinous nucleoplasm without structure (Seksek *et al.*, 1997). The exact process of compartment formation remains to be elucidated. It may result from the structural framework of the nucleus actively concentrating specific resident factors or, alternatively, the chance accumulation of components to form a nuclear architecture (Pederson, 1998; Misteli, 2001).

Before hypotheses as to compartment formation are presented, the dynamic nature of proteins themselves within the interchromatin space must first be outlined. Proteins have been shown to be highly mobile within the nucleus in an energy-independent, diffusional manner, as seen using fluorescently tagged native nuclear proteins (Houtsmuller *et al.*, 1999; Phair and Misteli, 2000; Shopland and Lawrence, 2000; Pederson, 2001). As the interchromatin space is relatively large compared to the space occupied by chromatin and the viscosity of the nucleus is similar to water (Fushimi and Verkman, 1991), diffusion may be an efficient means by which proteins distribute through the nucleus and find an appropriate binding site. This phenomenon, termed scanning (Misteli, 2001), does not require any specific targeting signals and seems to support this metabolically economic method of encountering a binding site.

This leads to the question, if proteins are highly mobile in nature, how do aggregations of proteins or compartments form? Proteins move slower than biologically inactive solutes of similar size in the nucleus. Using FRAP experiments, it has been shown that indeed movement of proteins occur at a slower rate than would be expected from their molecular weight (Kruhlak *et al.*, 2000; Phair and Misteli, 2000). Specific biological properties of the protein must be taken into account and protein impedance may be due to the binding of the protein into a very large complex or to a component that is relatively immobile.

Misteli presents a model for stochastic recruitment of proteins to their binding sites and compartment formation (Misteli, 2001). Steady-state compartments are the result of perceived influx and efflux of proteins from discrete interchromosomal areas. For the compartments that are not associated with chromatin, a relatively stable nucleation site for compartment formation, the appearance of discrete foci with set characteristics such as size and number may not be apparent. A stochastic, self-association mechanism is promoted as the foundation for compartment formation and foci are a result of binding to a complex of free factors that are more dynamically inhibited. He also attributes the apparent budding, changes in shape and fluorescence of SFC and CBs as the association and dissociation of factors (Misteli and Spector, 1997; Misteli *et al.*, 1998; Platani *et al.*, 2000). Another model proposed by Kruhlak *et al.* suggests that SFCs are composed of subpopulations of splicing factors (Kruhlak *et al.*, 2000). The apparent diffusional movement and budding of speckles is proposed to be a result of dynamic remodeling of the karyoskeleton, to which SFCs are known to associate (Hendzel *et al.*, 1998; Hendzel *et al.*, 1999). These are considered to be relatively immobile binding sites and, thus, a non-random organization of stable nucleating binding sites contributes to compartment nucleation. More dynamic subpopulations of SFCs can be attributed to regulation by phosphorylation (Misteli and Spector, 1997) and thus a calculation of kinetics for the general population of many factors in compartments may not easily be realized (Phair and Misteli, 2000; Pederson, 2001). In addition to this, the Sp100 component of PML bodies, which is a nuclear matrix-associated protein (Szostecki *et al.*, 1990), has recently been shown to be a non-dynamic component of the body and show PML bodies are positionally stability over time (Boisvert *et al.*, 2001 but see Muratani *et al.*, 2002). Not only may Sp100-enriched areas be nucleating sites for compartments, but also the relative positional stability contradicts Misteli's model of completely dynamic nuclear compartments with stochastic binding resulting in complex self-organization.

1.8 Subnuclear Organization of Transcription Factors and their Dynamics

It is characteristic for transcription regulatory proteins to distribute throughout the interchromatin space in several hundred discrete foci. This often, but not necessarily, occurs in a pattern within the interchromatin compartment reflective of the location of binding site clusters in permissive chromatin regions (Grande *et al.*, 1997; Hendzel *et al.*, 1998; Hendzel *et al.*, 2001). All HATs and most HDACs produce characteristic interphase foci. HATs localize to several hundred microdomains within the nucleus, enriched on the exterior of splicing factor-containing nuclear speckles, interior to surrounding hyperacetylated chromatin at these sites, and depleted at the nuclear periphery (Hendzel *et al.*, 1998; Figure 1.5, also from Hendzel *et al.*, 1998). The

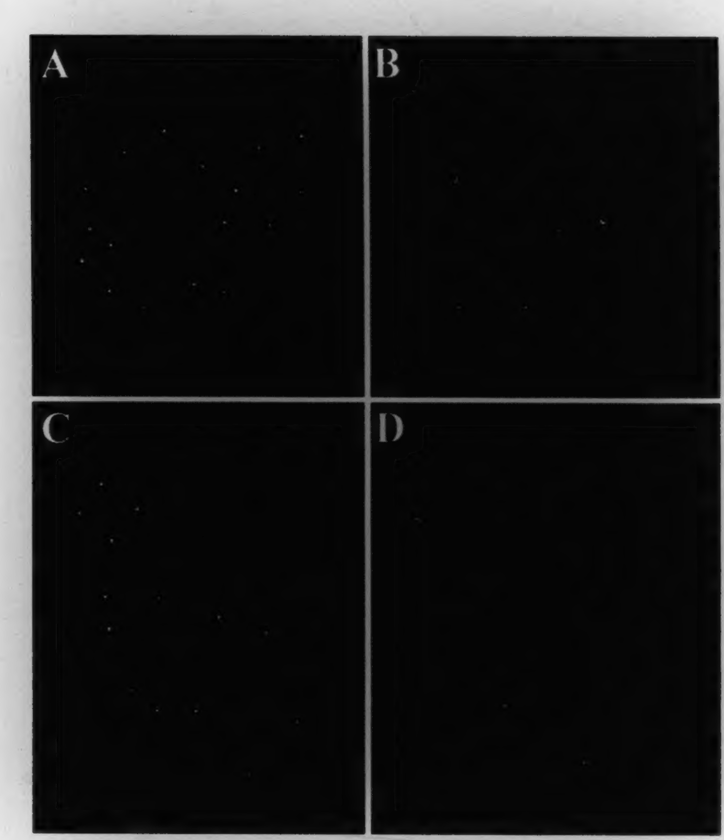

Figure 1.5 Non-random spatial distribution of acetyltransferase activity in the nucleus. Highly acetylated histone H3 (red) localizes on the periphery of splicing factor compartments (green) within intranuclear space, devoid of chromatin (blue). The interchromatin space is denoted by yellow dots in panels A and C. Activity is depleted in the condensed chromatin regions at the periphery of the nucleus in the nucleoli. HDAC inhibitor treatment (C, D), increases acetyltransferase activity. The increased acetylated H3 signal occurs in the same distribution pattern, indicating this activity is limited to the same subset of chromatin. Therefore, HATs and HDACs are preferentially active on the same subset of chromatin. Source: Hendzel *et al.*, 1998.

enrichment of these factors near nuclear speckles correlates with an enrichment in chromatin undergoing dynamic histone acetylation, a hallmark of transcriptionally active and competent chromatin. Frequently, PML bodies are neighbors of speckles. CBP is a dynamic component of PML bodies, which themselves have high positional stability along with factor enrichment in non-chromosomal space and are implicated in maintaining permissive chromatin (Boisvert *et al.*, 2000; Borden, 2002). Thus, there is a spatial relationship between the location of the enzymes involved in regulating histone acetylation and the location of chromatin undergoing reversible histone acetylation. This is despite the observation that the microdomains containing these factors appear to be physically adjacent rather than overlapping regions of highly acetylated chromatin (McManus and Hendzel, 2001). The localization of these enzymes in non-chromatin regions suggests that subnuclear targeting of these factors may be somewhat independent of chromatin. Dispersal of HATs and HDACs into interchromosomal spaces through mitosis correlates with *in situ* inactivation but not catalytic inactivation *in vitro*. Their return to foci in interphase correlates with the return of *in situ* reactivation, demonstrating a functional correlation with the formation of these foci (Kruhlak *et al.*, 2001). Therefore, the purpose of these foci could be to functionally concentrate specific nuclear proteins to facilitate the assembly of, and control global and local nucleoplasmic concentrations of, multimolecular complexes (Hendzel *et al.*, 2001).

Nucleation of compartments whose activities are specific to chromatin remodeling and transcription machinery is theorized to occur when a transcriptional activator, cis-regulatory factor or a component of transcriptional machinery associates transiently (scans) the chromatin and finds a lower energy binding site. The increased affinity for the appropriate site allows for a longer residence time on the chromatin. At this time, if no chromatin modifying activity is recruited within the residence time of the activator, through the same stochastic scanning mechanism, the activator dissociates and there is no activity. Transcriptional activators such as steroid receptors have shown to be dynamically exchanged on their response elements even in the presence of ligand (Hager *et al.*, 2000; McNally *et al.*, 2000; Becker *et al.*, 2002), which was previously thought to confer a permanent stability to the chromatin (Berk, 1999). Additionally, the requirement for regulation of a given locus may dictate residence times and dynamics of regulatory factors associated with RNAPII (Hager *et al.*, 2002). This exchange may be consistent with the Misteli model. What is not mentioned is that steroid receptors are known to associate with nuclear matrix proteins (DeFranco and Guerrero, 2000). The nuclear matrix is classically described biochemically as a structure that remains after the electrophoretic removal of chromatin from the nucleus under physiological conditions or following the salt extraction of nuclease-digested nuclei. In recent years, the nuclear matrix can be considered a dynamic structural framework composed of a web of constituent filament proteins linked to nuclear lamina proteins, necessary for the structural integrity of the nucleus (Davie, 1997). Variable regions of interphase thirty nanometer decondensed chromatin fibers, the structures upon which transcription occurs, are known to have matrix or scaffold attachment regions (MARs/SARs) encompassing clusters of cis-regulatory elements (Forrester *et al.*, 1994). MARs/SARs' proposed action is to be insulators between active and inactive chromatin (Namciu *et al.*, 1998). Recent work by Ostermeier *et al.* confirms this to be the case for the β-globin locus: the first evidence that nuclear matrix association dynamically mediates transcriptional control by altering the chromatin structure of the gene locus (Ostermeier *et al.*, 2003). Although not discrediting the stochastic "hit-and-run" mechanism of Misteli's, proponents of a dynamic nuclear matrix or karyoskeleton in transcriptional control, compounded with these recent findings, may deem that this mechanism is simplistic. This stochastic mechanism is also proposed for the assembly of the core transcriptional apparatus but again does not include an association with a nuclear matrix, which would better encompass the problem of the highly improbable formation of the transcription complex by providing sites of attachment to recruit factors and help target initiation (Bral *et al.*, 1998; Lemon and Tjian, 2000).

Recent studies into the temporal and dynamic nature of gene activation at various loci have challenged the theory that the RNAPII holoenzyme and transcriptional apparatus bind to chromatin as preassembled structural entities and that binding of these factors is not dynamic. Proponents of the preassembly model cite the advantage of a pre-formed holoenzyme that concentrates individual transcription factors with a limited cellular concentration (Parvin and Young, 1998). This view that does not adequately explain dynamic transcriptional regulation, steric challenges in gaining access to target promoters posed by chromatin structure, or differences in complexes required for initiation and transcription (Lemon and Tjian, 2000). However, it has been discovered through recent studies that specific temporal sequences of coactivator and regulatory complex recruitment to target promoters exist. Studies conducted *in vivo* show the dynamic nature of coactivator complex formation and the rapid exchange of factors with residency times contingent on function (Becker *et al.*, 2002; Dundr *et al.*, 2002). The culmination of a specific sequence of histone modifications, coupled with these various enzymatic activities, result in transcriptional initiation (Featherstone, 2002).

Of importance are the results from Belmont's group monitoring the *in situ* co-activator recruitment upon targeting of the VP16 acidic activation domain to a heterochromatic chromosome (Memedula and Belmont, 2003). Different components of both SWI/SNF and HAT complexes show different recruitment patterns, indicating possible assembly of chromatin-modifying complexes on the gene target rather than diffusing onto chromatin as intact entities. Interestingly, there is differential recruitment of various HATs, mirrored in the acetylation patterns of the locus; hyperacetylation of H4 slightly lags that of acetylated lysine 14 of H3 (Memedula and Belmont, 2003). Additionally, TBP binding and continued residency correlates closely with activator binding, rather than with histone acetylation (Katan-Khaykovich and Struhl, 2002).

Therefore, the order of factor recruitment may not be significant and this allows for multiple paths to result in the same activated state, stressing the need for only a population of factors concentrated at a locus. Temporal differences could significantly influence physiological outcomes or susceptibility to epigenetic influences (Belmont, 2003).

1.9 Herpes Simplex Virus I Infection in the Nucleus

HSV-1 is a DNA virus that utilizes only the host transcriptional machinery for gene expression and inhibits host cell transcription. Distinct viral replication compartments (VRCs) are established in defined regions within the nucleus that exclude host chromatin without physical barriers such as membranes, but do not themselves contain chromatin (Rixon *et al.*, 1983; Puvion-Dutilleul *et al.*, 1985; Randall and Dinwoodie, 1986; Spencer *et al.*, 2000). As the formation of replication compartments could influence nuclear processes through a steady-state depletion of nuclear machinery, it is reasonable to hypothesize HSV-1 might alter the subnuclear distribution of HATs and HDACs in order to facilitate the stable repression of the host genome. By imposing abnormal compartmentalization and disrupting normal nuclear compartments containing HATs and HDACs, changes in host chromatin histone acetylation are able to be correlated with transcriptional repression and altered subnuclear organization of HATs and HDACs.

HSV-1 is a class I double stranded DNA virus of approximately 152 kilobases encoding over eighty genes expressed sequentially in a temporal cascade based roughly on the time post-infection of their expression (Clements *et al.*, 1977; McGeoch *et al.*, 1988). The virus inhibits host cell transcription through the process of virion host shutoff (VHS; Kwong and Frenkel, 1987, 1989). This is initiated in the nucleus by the VHS protein that is introduced into the nucleus upon infection and results in the destabilization of host mRNAs (Smibert *et al.*, 1992; Zelus *et al.*, 1996) and the aberrant phosphorylation of host RNA Polymerase II (Rice *et al.*, 1994; Rice *et al.*, 1995). This potentially contributes to the observed host transcriptional shut down late in the lytic cycle.

The lytic cascade of HSV-1 genes offers rigid parameters within which productive infection can occur, providing a system to dissect mechanisms involved in the preferential activation of specific RNAPII genes in mammalian cells, where genes are in abundance and regulatory proteins are limiting. Viral gene expression proceeds in the order of immediate-early genes (IE), whose five gene products (ICP0, ICP4, ICP22, ICP27 and ICP47) appear within minutes of infection and stimulate subsequent viral gene transactivation, early genes (E), whose products are required for replication of the viral genome and late gene expression, and late genes (L) that encode structural and regulatory proteins.

The virion tegument protein VP16 (αTIF), a potent eukaryotic transcriptional activator and gene product of the late (L) genes, transported to the nucleus along with the HSV-1 genome, facilitates the transactivation of immediate-early (IE) viral genes. VP16, aided by the actions of VHS (Lam *et al.*, 1996), is the source of the initial transcriptional selectivity due to its conserved DNA binding surface that recognizes cis-regulatory sequence elements on viral DNA and orchestrates the assembly of a transcriptional regulatory complex (Babb *et al.*, 2001). However, the specific mechanisms that distinguish and preferentially transcribe viral genes amongst a large excess of host genes are not well defined. While there is substantial evidence for promoter sequence recognition and spatial organization and recruitment of factors as a means for preferential generation of IE viral transcripts, the mechanisms targeting the induction of E and L genes over host genes are largely unknown. HSV-1 and host promoter sequences are indistinguishable and there is no evidence that primary nucleotide sequence of viral promoters is responsible for favored activation of E and L genes (Cheung *et al.*, 1997). Viral promoters containing an isolated TATA sequence, found ubiquitously in eukaryotic promoters and outnumbering the virus by orders of magnitude, are somehow preferably induced by the IE protein ICP4 (Homa *et al.*, 1988; Kibler *et al.*, 1991; Imbalzano and DeLuca, 1992; Cheung *et al.*, 1997). ICP4 has been shown to interact with members of the basal transcription apparatus including TATA Binding Protein, TFIIB and TAFII250, a histone acetyltransferase (Smith *et al.*, 1993; Carrozza and DeLuca, 1996). Localization of HAT activity through viral infection may be of importance. Treatment of virally infected cells with the potent histone deacetylase inhibitor Trichostatin A (TSA) resurrects ICP0 mutants and aids in creating productive infections from some latent neuronal cultures (Hobbs and DeLuca, 1999; Arthur *et al.*, 2001)

, emphasizing the need to control global acetyltransferase activity to allow viral transcription.

While HSV-1 processes are temporally regulated by the cascade of viral gene expression, there is an additional level of spatial control imposed within the nucleus wherein nuclear organization is altered, normal distribution of factors like nucleoli are disrupted and chromatin is marginalized (Darlington and James, 1966; Sirtori and

Bosisio-Bestetti, 1967; Schwartz and Roizman, 1969). First, the virus establishes prereplicative foci whose location is defined by host cell architecture (de Bruyn Kops and Knipe, 1994). These foci are associated with and establish adjacent to nuclear matrix-associated PML bodies via a targeting mechanism independent of viral protein expression (Ishov and Maul, 1996; Maul *et al.*, 1996; Everett *et al.*, 2003). Early transcription of the viral genome also takes place on the periphery of the PML body and successful development of viral replication compartments is contingent upon both viral and native PML body protein factors (Maul, 1998; Hsu and Everett, 2001; Tang *et al.*, 2003). The virus, through colocalization of the ICP0 gene product to PML bodies, disrupts and destroys them by inducing proteasome-dependent degradation that is unnecessary for productive viral infection (Everett, 2001; Lopez *et al.*, 2002). However, despite this disruption, evidence indicates that the interaction between viral genomes and factors associated with PML could affect the outcome of a viral infection at its very earliest stages, and thus nuclear localization adjacent to PML may be more than coincidental (Lukonis *et al.*, 1997; Lukonis and Weller, 1997). Although thought to be implicated in host antiviral response, and perhaps more importantly for viral replication, the PML body may localize and concentrate nuclear factors needed in establishing a transcriptional domain (Boisvert *et al.*, 2000). CBP, the transcriptional coactivator and acetyltransferase, is a dynamic component of PML bodies. In addition to concentrating factors needed for transcription, disruption of these bodies could alter the level of transcriptional activity in surrounding host genes (Boisvert *et al.*, 2001) and normal acetylation patterns. The coincident presence of transcriptional repressors, including HDAC2, to the nuclear site of viral transcription *in vivo* remains unexplained.

Additionally, cells over expressing PML do not show an increase in productive prereplicative foci (Lopez *et al.*, 2002), possibly indicating that the native PML body environment and factor dynamics are required for the proper execution of normal viral processes. This may not be reproduced in cells aberrantly expressing PML.

Nuclear organizational domains other than nucleoli and PML are affected by HSV-1 infection. As HSV-1 has few introns, host splicing factors may be used minimally by the virus. IE genes cause the redistribution of the small nuclear ribonucleoprotein particles (snRNPs), constituent factors of IGCs or speckles, to change the normal foci pattern to a restricted punctate distribution with a concomitant loss of coiled bodies, similar to that seen in transcriptionally inhibited cells (Phelan *et al.*, 1993).

Although largely unexplored, there are indications that viral gene products act to interfere with some chromatin remodeling processes, including those associated with histone acetylation as mentioned previously, on the host chromatin. ICP4 and ICP0 may act to either break or bypass the chromatin-dependent silencing mechanism of α–globin genes in non-erythroid cells (Cheung *et al.*, 1997). Also, the tegument protein VP22, shown to associate with histones and nucleosomes, inhibits nucleosome deposition on DNA by binding to the host proteins TAF-I, a chromatin remodeling protein and component of the inhibitor of acetyltransferases (INHAT) complex. However, its function of inhibiting the histone acetyltransferase activity of p300 or PCAF, is not compromised *in vitro*, suggesting the possibility of spatial reorganization as a means of control (Van Leeuwen *et al.*, 2003). This association with histones is also seen as acetylation is decreased in cells expressing the tegument protein of VP22 from bovine HSV-1, although no HAT or HDAC activity data was reported (Ren *et al.*, 2001).

Most studies have focused on the characterization of the viral compartment, its constituents and its association with host proteins in the context of the viral infection and proliferation. Little is known of how HSV-1 reorganizes nuclear architecture or chromosome territories through the growth of its viral replication compartments. Recent work by the Sullivan group (Monier *et al.*, 2000; N. MacLean, unpublished observations), has quantitatively characterized the structural alterations of the host chromatin and interchromatin space in live cells using H2B-GFP as the chromatin marker. The viral replication compartment itself is a process-dependent domain. This was demonstrated by the treatment of cells with phosphonoacetic acid, a viral DNA polymerase inhibitor, which prevented the formation of VRCs. This confirms previous observations that VRCs are functional compartments in the nucleus dedicated to the replication of viral DNA and viral assembly (de Bruyn Kops and Knipe, 1994). Monier *et al.* (2000) showed that the formation of VRCs is due to a redistribution of chromatin and therefore must be depleted in the region by compaction or migration away from VRCs in a manner that has not been determined. The group identified three stages in the host nuclear response to viral infection using the criteria of nuclear morphology and compartment function. In the first stage, annexation, the existing interchromatin space is consolidated by the virus and there is no visible change in the host chromatin architecture, despite the fact that functional activities of the nucleus have been switched to viral replication. Indeed, a recent *in vivo* study into intranuclear movement of viral replication structures using ICP8-GFP shows annexation by coalescence of smaller distant prereplicative foci into larger compartments by movement to neighboring foci. Annexation also occurs when larger compartments grow by accretion, maintaining relative positional stability, until eventually compartments fill the nucleus (Taylor *et al.*, 2003). This, the VRC expansion phase, is characterized by the presence of well-developed chromatin depleted regions and the spatial rearrangement of the host chromatin to the periphery of the nucleus (Monier *et al.*, 2000). This expansion is coincident with a doubling of nuclear volume to accommodate the VRCs, without the compaction of chromatin, and indicates that the chromatin is most likely still in its native state and theoretically accessible by associated enzymes. Nucleoli remain intact through this stage. Very late in the infection process, the host chromatin volume is compacted linearly over time until lysis. Chromatin depletion in the VRCs is speculated to be due to the mutual exclusion property of a phase separation phenomenon (Tjerneld and Johansson, 2000) or a mutual local increase of interacting molecules and a self-assembly property of the compartment that excludes chromatin as an incompatible structural component (Hecht *et al.*, 1995). There is also speculation that VRC expansion may be due to large scale chromatin movement imposed by transcriptional modulators that reprogram the intranuclear positioning of chromosome loci in a cell cycle-dependent manner (Tumbar and Belmont, 2001; Belmont, 2003; Taylor *et al.*, 2003). More intriguingly, it is speculated that nuclear motors may be the underlying cause of prereplicative loci translocation and integral to the expansion of VRCs (Taylor *et al.*, 2003). Understanding viral compartment formation, as a functional compartment imposed on the normal organization of the nucleus, will provide insight into the principles involved in the assembly and function of the compartmentalized nucleus.

2. MATERIALS AND METHODS

2.1 Cell Culture and Viral Infection of Adherent Cells for Microscopy

Adherent cell lines (Indian Muntjac [IM] fibroblast, SK-N-SH neuroblastoma, Human Intestinal Smooth Muscle [HISM], HeLa, COS-1 and Vero Green Monkey kidney cells) were grown directly on No. 1 (0.17 mm thickness) glass coverslips at 37 °C and 5% humidity in conditions recommended by the American Type Culture Collection (Manassas, VA). To assay for the recruitment of endogenous and transfected expressed proteins, cells were seeded and grown to approximately 70% confluence so as to be in the normal growth phase for productive infection. To test for recruitment of transiently expressing proteins, adherent cells were transfected on cover slips 12-24 hours prior to infection. GFP- and Flag-conjugated expression vectors were prepared as instructed (Clonetech). CBP-GFP vector constructs (kindly provided by Kirk McManus) were transfected into IM and HISM cells and stably transfected into COS cells, AP-2-GFP vector constructs were transfected and also stably transformed into HeLa cells and empty GFP vector constructs were transfected into HeLa and IM cells. Stably expressing estrogen receptor-GFP MCF-7 cells and vector constructs of ER-GFP for transfection into HISM cells were kindly provided and characterized by Jim Davie (Zhao *et al.*, 2002). All HDAC constructs were kindly provided and characterized by Wolfgang Fischle (Fischle *et al.*, 1999; Fischle *et al.*, 2001) and prepared as described (Verdel and Khochbin, 1999). HDAC1 vector constructs were transfected into SK-N-SH, IM, COS and HISM cells. All other HDAC vector constructs were transfected into SK-N-SH, IM and HISM cells. Transfections were performed for each cover slip by combining 4 µl of

LipofectAMINE transfection reagent (Invitrogen) with 2 µg DNA in 200 µl serum free media, incubating at room temperature for 45 minutes, and then diluting to 1 ml with serum free media. Regular media was removed from the cover slip wells, rinsed with 1X PBS and replaced with the prepared transfection media. Cover slips were incubated for 5-8 hours at 37 °C and then replaced with regular media and allowed to recover for an incubation period of 12-24 hours pre-infection as infection rates are decreased in newly-transfected cells (MacLean, unpublished observations).

Herpes Simplex Virus type 1 (HSV-1) wild-type strain KOS1.1 stock solution (provided by Charlotte Spencer) was made and titers were obtained as previously described (Rice *et al.*, 1994). Cells were infected at a multiplicity of infection (MOI; ratio of viral particles to target cells) of 10 plaque-forming units (PFU) per cell as described (Rice *et al.*, 1995). Briefly, viral stock was added to PBS infection buffer (1% sterile glucose solution, 1% heat inactivated fetal bovine serum, 900µM $CaCl_2$, 500µM $MgCl_2$, in 1X PBS) and gently homogenized with a pipette. Growth media was replaced with viral solution and cells were incubated 50 minutes. The buffer was then removed and replaced with viral growth media (2% heat inactivated fetal bovine serum, 1X penicillin/streptomycin, 2 mM glutamine in DMEM) for continued incubation.

2.2 Cell Fixation and Immunofluorescent Staining

Infected and non-infected coverslips were immersed and fixed for a minimum of 5 minutes with 4% paraformaldehyde in 1X PBS (pH 7.5). Fixation instantly stops the infection process. Cells were washed three times with PBS and permeabilized by incubating in 0.5% Triton X-100/PBS for 5 minutes. Subsequently, they were rinsed with 0.1% Triton X-100/PBS and twice with 1X PBS. Coverslips were then blocked for 30 minutes in 1% PBS skim milk powder solution at room temperature. Meanwhile, primary antibody solutions were prepared in skim milk block and divided into 10 μl aliquots per coverslip onto parafilm. Anti-ICP4 monoclonal antibody (H1101, Goodwin Institute, 1:2000 dilution) was used as the indicator for infections as the protein is expressed within minutes of infection and persists within viral compartments until lysis. Coverslips were again rinsed with 0.1% Triton X-100/PBS, then rinsed in 1X PBS and inverted onto the antibody aliquots to incubate for 60 minutes at room temperature. The rinsing procedure was repeated and coverslips were incubated at room temperature for 30 minutes on parafilm with secondary antibody aliquots diluted to 1:200 with 1X PBS. The primary antibodies used for testing recruitment to viral compartments were commercially supplied by: Upstate Biotechnology (CBP-C terminus and CBP-N terminus, p300 monoclonal and powerclonal that recognizes CBP and p300, PCAF, ACTR, MYST, HDAC1, HDAC4, acetyl lysine, acetylated K12 of H4), Santa Cruz Biotechnology (TAFII250, AP-2, mSIN3A), BD Transduction Laboratories (CREB/CREM, $\mathrm{PKA}_{\mathrm{RIA}\alpha}$ Protein Kinase A type-1 alpha regulatory subunit), New England Biolabs (phosphorylated serine 15 of p53, HDAC5), Oncogene Research Products (Rb Ab-7), and Sigma (anti-FLAG tag). Additional primary antibodies were kindly provided by Wolfgang Fischle (HDACs 1, 2, 3, 4, 5 and 7), Gordon Chan (ATM), and J.B. Rattner (NuMa, Topoisomerase IIα). The monoclonal antibody to SC-35 was used directly as cell culture supernatant (Hendzel *et al.*, 1998). Depending on the primary antibodies used, the secondary antibodies that were used for detection included Cy3 donkey anti-mouse or rabbit immunoglobulin G (IgG), Cy5 donkey anti-mouse or anti-rabbit IgG

(Jackson ImmunoResearch), Alexa 488 goat anti-mouse or anti-rabbit IgG and Alexa 594 goat anti-human IgG (Molecular Probes). Controls for cross-reactivity of secondary antibodies and infected cells without primary antibodies were negative in the nucleus. Coverslips underwent the rinsing procedure again and were then mounted on glass microscope slides with a 90% glycerol-PBS (pH 7.5)-based medium containing 1 mg/ml paraphenylenediamine and 1 µg/ml 4',6 –diamidino- 2- phenylindole (DAPI), a fluorescent dye that selectively binds chromatin.

2.3 Immunofluorescent Imaging

A Zeiss Axioplan IIM epifluorescent digital imaging microscope (DIM) equipped with a 12-bit CCD camera (Sensicam, Cooke Corp.) using Metamorph imaging software (Universal Imaging Corp.) was used to elucidate the recruitment of proteins to viral compartments. Imaging requiring quantification of pixel data was performed on a Zeiss LSM 510 confocal microscope, equipped with a 40X (1.3NA) and 63X (1.4NA), using four channels (DAPI/364 nm UV laser, Alexa 488/488 nm Argon laser, Cy3/543 nm HeNe laser, Alexa 594 or Cy5/633 nm HeNe laser) and recorded as 12-bit digital images. False coloring and superimposition of images for figures were performed offline using Adobe Photoshop.

2.4 Immunodetection of Nascent RNA Transcripts in HSV-1 Infected Populations

To follow host transcription through HSV-1 infection, SK-N-SH cells were cultured on glass cover slips for indirect immunofluorescent analysis and infected as described. At 5 hours post-infection, 5-fluoruridine was added to the viral growth media to a final concentration of 1 mM for 15 minutes. The media was replaced and the infection allowed to progress for an additional hour before fixation. This incubation period was adequate to ensure all levels of infection, as well as uninfected cells, were represented in the cell population. Samples were permeabilized and blocked as described. Coverslips were again rinsed with 0.1% Triton X-100, then 1X PBS and applied to a solution of monoclonal anti-5-bromo-2'-deoxyuridine (anti-BrdU; Sigma) IgG at a 1:1000 ratio in 1% PBS skim milk solution for 60 minutes at room temperature to label halogenated UTP. Coverslips were rinsed and incubated with Alexa 488 goat anti-mouse IgG (Molecular Probes) for 30 minutes. Coverslips were rinsed, fixed again to ensure target epitope-antibody crosslinking and to eliminate crossreactivity, permeabilized and blocked as described. H1101 ICP4 primary antibody, to label viral compartments and assess infection level, and secondary Cy3 donkey anti-mouse IgG (Jackson ImmunoResearch) were applied as described. In an additional experiment using rabbit anti-acetylated K8 of H4 IgG (Upstate Biotechnology) to delineate viral compartments, primary antibodies were combined, cells were costained and secondary Cy3 donkey anti-rabbit IgG (Jackson ImmunoResearch) was applied as described without separate antibody application and no additional fixation. Coverslips were mounted on glass microscope slides using 90% glycerol-PBS (pH 7.5)-based medium containing 1 μg/ml DAPI. 12-bit confocal cell field images and individual cell images were taken and tests for cross-reactivity of mouse epitopes, determined to be negative, were performed using the colocalization function in the LSM 510 software.

2.5 Histone Isolation and Acid Urea Triton X-100 (AUT) Gel Electrophoresis

To resolve histones and their acetylation *in vitro*, HeLa S3 cells were cultured in suspension as recommended by the ATCC in spinner flasks in DMEM media with 10% heat inactivated serum. In log growth phase, cells were counted (Coulter) and half the volume was removed to serve as the uninfected control. The remaining cells were infected at a density of $1X10^6$ cells/ml. Viral stock was added to the growth media to infect the cells at an MOI of 10. At 6 hours post-infection, both infected and non-infected cells were spun at 1500 rpm for 10 minutes at 4 °C. Supernatant was poured off and cells were washed and spun three times with 10X volumes of cold 1X PBS. Cells were then frozen at –80 °C. As controls, small volumes of both cell populations were reserved, placed on poly-l-lysine treated cover slips for 15 minutes to adhere the cells at 1 mg/ml (Sigma), fixed and stained as described with anti-ICP4 to check for infection level and contamination. Histones were isolated using an acid extraction method (Nickel *et al.*, 1987) at 4 °C. Cells were resuspended in 10X volume of Dignam buffer A (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT added fresh, 1 mM PMSF added fresh), dounce homogenized and spun at 3000 g for 10 minutes. The pellet was resuspended in Buffer A and 0.4 N H_2SO_4 added drop wise and left on ice for 30 minutes. The extract was spun at 1200 g for 10 minutes at 4 °C and the supernatant was dialyzed versus 0.1 N acetic acid and 2 changes of ddH_2O at 4 °C. The solution was then frozen, lyophilized and dissolved in 50 μl of AUT gel loading dye (100 mM tris-acetate pH 8.8, 20% glycerol, 8 M urea , 5% β-mercaptoethanol, 2% thiodiglycol, pinch of Pyronin Y dye) under a fume hood.

AUT gels were performed as described (Bonner *et al.*, 1980) with a few modifications. Briefly, a 15% separating gel (acrylamide [29:1], 1%TEMED, 40% urea [w/v], 10% 0.004% riboflavin solution, 0.3 M Triton-X 100, 1% thiodiglycol) was made fresh and heated in the dark to avoid polymerization. It was then poured, layered with water saturated n-butanol, polymerized in front of light box for 30 minutes and placed in 4 °C overnight. The butanol was wicked away, stacking gel poured (acrylamide [29:1], 4% TEMED/43.1% acetic acid, 40% [w/v] urea, 0.0004% riboflavin, 3 M potassium acetate, 0.3M Triton-X 100, 1% thiodiglycol, in dark conditions) and polymerized with light as above. Gels were run at 4 °C ambient temperature in 0.1 N acetic acid made fresh. Gels were stained with Coomassie Brilliant Blue (Amersham), imaged with a flatbed scanner and analyzed using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). Gel scans were normalized for background.

2.6 *In situ Acetylation, Acetyltransferase and Deacetylase Assays*

Indian Muntjac cells, due to their flat morphology and relatively synchronous infection, were used for immunofluorescent assays. Cells were grown on 12 mm diameter No. 1 glass cover slips in 24 well tissue culture plates. Cells were infected as described and fixed at intervals of 2, 4 and 6 hrs post-infection to follow approximately the infection cycle from early prereplication compartments to late formation of a large replication compartment. Cells for the HDAC activity assay were fixed at 3, 6 and 9 hours to assure the formation of larger, established replication compartments (high infection level) as I have observed a retardation in the infection process due to

Trichostatin A, a reversible histone deacetylase inhibitor, when added to the viral infection buffer (MacLean, see Figure 3.17). To evaluate HAT activity, cells were treated with the TSA at 0.1 μg/ml 1 hour prior to fixation. To assay HDAC activity, cells were treated with 0.1 μg/ml TSA 12 hours prior to infection, added to the infection buffer and viral growth media, and replaced with fresh media 1 hour prior to fixation to allow for HDAC recovery. The immunofluorescent antibodies tested were anti-penta-acetylated hv1where one or combination of K16, K12, K8, K5, and K20 of H4 are acetylated (Hendzel *et al.*, 1997), anti-AcH3 where both lysines 9 and 14 are acetylated (Boggs *et al.*, 1996; Upstate Biotechnology), anti-H2B acetylated at K5, K12, K15 and K20 (Belyaev *et al.*, 1996; Serotec) and anti-acetylated H4 at K8 (Turner *et al.*, 1989); Upstate Biotechnology). Dilutions were 1:1000, 1:600, 1:800 and 1:8000 respectively, optimized through trials using various antibody dilutions to minimize confocal signal saturation and nonspecific cytoplasmic binding. Random 12-bit confocal field images were taken at constant laser, microscope and detector settings, optimized for each experiment, to allow comparison between samples. Metamorph (Universal Imaging Corp.) was used to analyze image data. Briefly, a median filter was applied to each image in order to reduce noise (3X3 and 1 iteration) and cell masks were made by thresholding using the DAPI channel to delineate the nucleus. Total gray values for chromatin and acetylation images were exported into Microsoft Excel spreadsheets. Also exported was the average gray value for a 50X50 pixel square traced for background measure. Data from partial cells, mitotic cells, overlapping nuclei, objects less than 400 pixels (40X, 1.3NA) and saturated pixels were excluded. Corrected gray values were obtained by subtracting the total area multiplied by the background area from the gray value for each image. The acetylation level is the amount of acetylation for the amount of chromatin per cell. Cells were scored for three categories of infection based on ICP4 stain. Low infection is classed by a discernable ICP4 stain to the formation of small distinct punctate prereplicative sites. Not all prereplicative sites are productive and medium level infection is the establishment and expansion of a few prereplicative sites to larger replication compartments. High levels of infection are classed when replication compartments annex and form very large or single replication compartments, usually observed at approximately 50% nuclear occupancy or greater by area in optical sections through the approximate center of the cell. Descriptive statistics were compiled for each experiment within each category and the means were compared between low, medium and high infection levels using unpaired t-tests (Graphpad.com).

2.7 Histone Immunoblotting

Indian Muntjac cells were seeded identically and grown in T_{175} flasks and infected as described for 4, 6, and 8 hours to approximate infection levels seen for the *in situ* acetylation assay. Staining fixed cells at the same time points with ICP4 allowed for the evaluation of infection levels. The cells were trypsinized at the completion of their post-infection incubation to stop infection. The cells were washed and the histones were extracted by the acid extraction method as described for the AUT gels. SDS-PAGE gels (12% acrylamide) were run and the electrophoretically separated proteins were stained with Coomassie Brilliant Blue and transferred or transferred to Hybond-C paper. Immunoblotting was performed using an ECL system from Amersham. Blocking and antibody incubations used 5% milk powder in TBS-Tween (20 mM Tris-HCl, pH 7.2-7.4

at room temperature, 150 mM NaCl, 01% [v/v] Tween 20) while washes used TBS-Tween. The primary antibodies used were AcH3, AcH2B and K8 as described above and used at dilutions of 1:1000, 1:400 and 1:2500 respectively. The secondary antibody was goat anti-rabbit HRP conjugated (Jackson ImmunoResearch) used at a 1:60000 dilution. SDS-PAGE and immunoblotting was performed by Dr. Christi Andrin.

2.8 In Vitro HDAC Assay

To corroborate levels of HDAC activity of *in situ* assays, an *in vitro* HDAC activity assay with infected cells was performed (Emiliani *et al.*, 1998). Suspension HeLa S3 cells were cultured as recommended by the ATCC in spinner flasks in DMEM media with 10% heat inactivated serum. In log growth phase, cells were counted (Coulter) and infected at a density of $1X10^6$ cells/ml. Viral stock was added to the growth media to infect the cells at an MOI of 10. An equal volume of cells was removed at 2,4,6,9 and 12 hours and spun at 1500 rpm for 10 minutes at 4 °C. Supernatant was poured off and cells were washed and spun three times with 10X volumes of cold 1X PBS. Cells were then frozen at –80 °C. As controls, small volumes of cells were collected at each time point, placed on 1 mg/ml poly-l-lysine (Sigma) treated cover slips for 15 minutes to adhere the cells, fixed and stained as described with anti-ICP4 to check the normal progression of infection through time. Cells for crude enzyme extracts were thawed at 4 °C in lysis buffer (50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 0.5 mM EDTA, 10% glycerol, 0.5% NP-40, 3 M urea, 1% 2-mercaptoethanol and 1X Protease Inhibitor Cocktail [Sigma] added fresh; recipe kindly provided by Dr. Wolfgang Fischle) at a ratio of 2.5 volumes of buffer for 1 ml of wet cells. Whole cells enzyme extracts showed negligible difference in HDAC activity to nuclear enzyme extract preps (Fischle *et al.*, 1999). Cells were dounce homogenized and spun at 10 000 g for 30 minutes at 4 °C. The supernatant was dialyzed in phosphate buffer (pH 7.2) and then subsequently in ddH_2O at 4 °C. Extracts were kept on ice and used immediately. Protein concentration was measured spectrophotometrically (Beckman) using the formula [mg/ml] = $(1.55XA_{280})$- $(0.76XA_{260})$ when the path length equals 1. The HDAC activity assay was performed as described (Hendzel and Davie, 1991; Fischle *et al.*, 1999). Briefly, 0.09 μg of protein extract was added to HD-buffer (10 mM Tris-HCl, pH 8.0, 10 mM NaCl, and 10% glycerol) to a total volume of 200 μl. 10 μl ^{3}H-histone substrate (kindly provided by Jim Davie) was added, lightly vortexed, and incubated for 45 minutes at 37 °C. Trials were typically done in triplicate. TSA was added to 400 nM to the HD-buffer and the enzyme mixture, placed on ice 45 minutes before incubation with the substrate to account for ^{3}H acetylation due to proteolysis. Blank samples were included as controls. Non-enzymatic release of label was usually below 100 dpm. 50 μl of stop mix (1M HCl, 0.16 M HAc) was added and vortexed to stop the reaction. 300 μl of ethyl acetate was added, vortexed and spun at 14 000 g for 7 minutes. 200 μl of the organic layer was extracted, added to 5 ml of scintillation fluid and dpm from the liberated acetate was counted (Beckman Coulter). The assay was performed in triplicate and the results were graphed using Prism (Graphpad).

2.9 Recruitment of HATs and HDACs

To test for preferential recruitment of either HATs or HDACs to viral compartments, SK-N-SH cells were cultured as described for immunofluorescence and infected and fixed at 3, 6 and 9 hour intervals to ensure the formation of distinct prereplicative and productive viral compartments. They were stained with HDAC1 (1:100 dilution; kindly provided by Wolfgang Fischle), anti-ICP4 (1:2000), and either anti-p300 monoclonal recognizing only the C-terminus of p300 or a powerclonal antibody recognizing a common epitope for both the C-terminus of p300 and CBP, both at the dilution of 1:200 (Eckner *et al.*, 1994); Upstate Biotechnology). Staining required the application of HDAC1 and p300 antibodies and complimentary Cy5 anti-rabbit and Cy3 anti-mouse secondary antibodies respectively. The application of anti-ICP4 required the subsequent fixation of cells for 5 minutes in 4% paraformaldehyde in 1X PBS, repermeabilization of cells with treatment with 0.5% TritonX-100 for 5 minutes, and application of anti-ICP4 and the secondary goat anti-mouse Alexa 488 as previously described. Control experiments omitting anti-ICP4 were negative for cross-reactivity with p300 after the second paraformaldehyde treatment was performed. High resolution 12-bit confocal images of one to three cells per field and at various viral compartment stages were taken (512X512, 4.6 zoom factor, 63X 1.4NA). Additional tests for cross-reactivity were performed using the colocalization function in the LSM 510 software as described. Metamorph (Universal Imaging Corp.) was used for data analysis and export to Microsoft Excel. Briefly, a median filter was applied to all images to reduce noise and threshold masks were made in the DAPI channel for total nuclei area and gray value measurements in the HDAC1 and p300 channels. Threshold masks of viral compartments were made in the ICP4 channel and area measurements were taken. With this mask both total gray and area measurements were made for the HDAC1 and p300 channels. 50X50 pixel background measurements were made in the HDAC1 and p300 channels. In Excel, gray values for total nuclei and viral compartments in the HDAC1 and p300 channels were corrected by using background measurements multiplied by the total area for each object and subtracting the product from the total measured gray value. The total immunofluorescent signal contained within viral compartments was obtained by dividing the gray value for signal within the viral compartments by the total gray value of the nucleus. The area of the nucleus occupied by viral compartments was obtained by dividing the area of viral compartments in a nucleus by the area of the nucleus. This can be represented mathematically by:

$$(\text{fluoresence}_{\text{compartment}}/\text{fluoresence}_{\text{total}})/(\text{area}_{\text{compartment}}/\text{area}_{\text{total}})$$

Data was exported to Prism (GraphPad) and the percentage of immunofluorescent signal for each nucleus was graphed versus the percentage of the nucleus occupied by viral compartment. The relationship was statistically determined to be linear. Preferential recruitment was defined as having a slope > 1 and a paired t-test statistical analysis of slope versus the m=1 standard was performed.

2.10 Fluorescence Loss in Photobleaching (FLIP) and Fluorescence Recovery after Photobleaching (FRAP)

IM and HISM cells transfected with CBP-GFP and infected with HSV-1 as described were placed on glass slides containing several drops of media surrounded by vacuum grease to form an airtight seal. Hoechst dye at 1 μg/ml was added to the media to label DNA for visualization of nuclei to identify infected cells and confirm the size and position of viral replication compartments. Live cell microscopy for photobleaching was performed using either the Zeiss LSM 510 laser-scanning confocal microscope with a 15 mA argon laser or a two photon multi-photon excitation (MPE) Zeiss NLO510 system with a Coherent Ti:Sapphire Mira900 femtosecond laser pumped with a 5 W Verdi laser (for Hoechst visualization to reduce phototoxicity) and a 15 mA argon laser. A 40 X 1.3 N.A. objective was used for all photobleaching experiments. Preliminary GFP imaging was performed with the Argon laser set to scanning mode to assess the initial imaging conditions. For FLIP, a small region in the centre of the nucleus was selected. The loss of fluorescence was monitored in the nucleus at no greater than 1% laser power in between repetitive bleach pulses (10 iterations at 100% laser intensity with 5 second intervals) with short 5 second intervals of recovery that allow for fluorescence redistribution. During the recovery intervals, cells are imaged with one scan at low-intensity illumination (1% laser power) to monitor the fluorescence redistribution. Recovery was considered complete when the intensity of the photobleached region stabilized. For FRAP analysis, laser intensities for initial evaluation of experimental conditions, photobleaching and cell imaging were maintained as with FLIP. Bleaching areas were selected, bleached and allowed to recover. The recovery period was monitored with consecutive low-intensity imaging scans until the fluorescence recovery was complete.

3. RESULTS

3.1 Histone Acetyltransferases and Potentially-Associated Proteins are recruited to Viral Compartments

Infection with HSV-1 results in the establishment of prereplicative foci around PML bodies and a recruitment of RNA polymerase II and factors associated with the initiation complex (Rice *et al.*, 1994; Jenkins and Spencer, 2001). As a prerequisite to further studies using HSV-1 as a model system for subnuclear reorganization of acetylation, it must first be determined if histone acetyltransferases and histone deacetylases are recruited to viral compartments. In order to test this, several different cell types were infected from both human and nonhuman species and examined for the distribution of histone acetyltransferases at different points post-infection. TAFII250, a component of RNAPII promoter-associated complexes and a known requirement for HSV-1 gene expression, is recruited to the viral compartments (Batterson and Roizman, 1983; Figure 3.1). It is shown here as a representative example of experimental results for the *in situ* HAT recruitment assay using indirect immunofluorescence. Panel A is a field view of SK-N-SH neuroblastoma cell nuclei with non-infected cells through to highly infected cells represented. Uninfected cells show normal interchromatin distribution patterns for TAFII250 (green) in interphase cells (Hendzel *et al.*, 1998), generally diffuse with punctate foci in regions distinct from condensed heterochromatic regions (blue). As HSV-1 does not package its DNA into chromatin (Spencer *et al.*, 2000), established viral compartments are clearly distinguished as non-nucleolar clearing in the nucleus (B). The identification of these regions as viral compartments was confirmed by costaining with the HSV-1 immediate early protein ICP4 (C). Infected

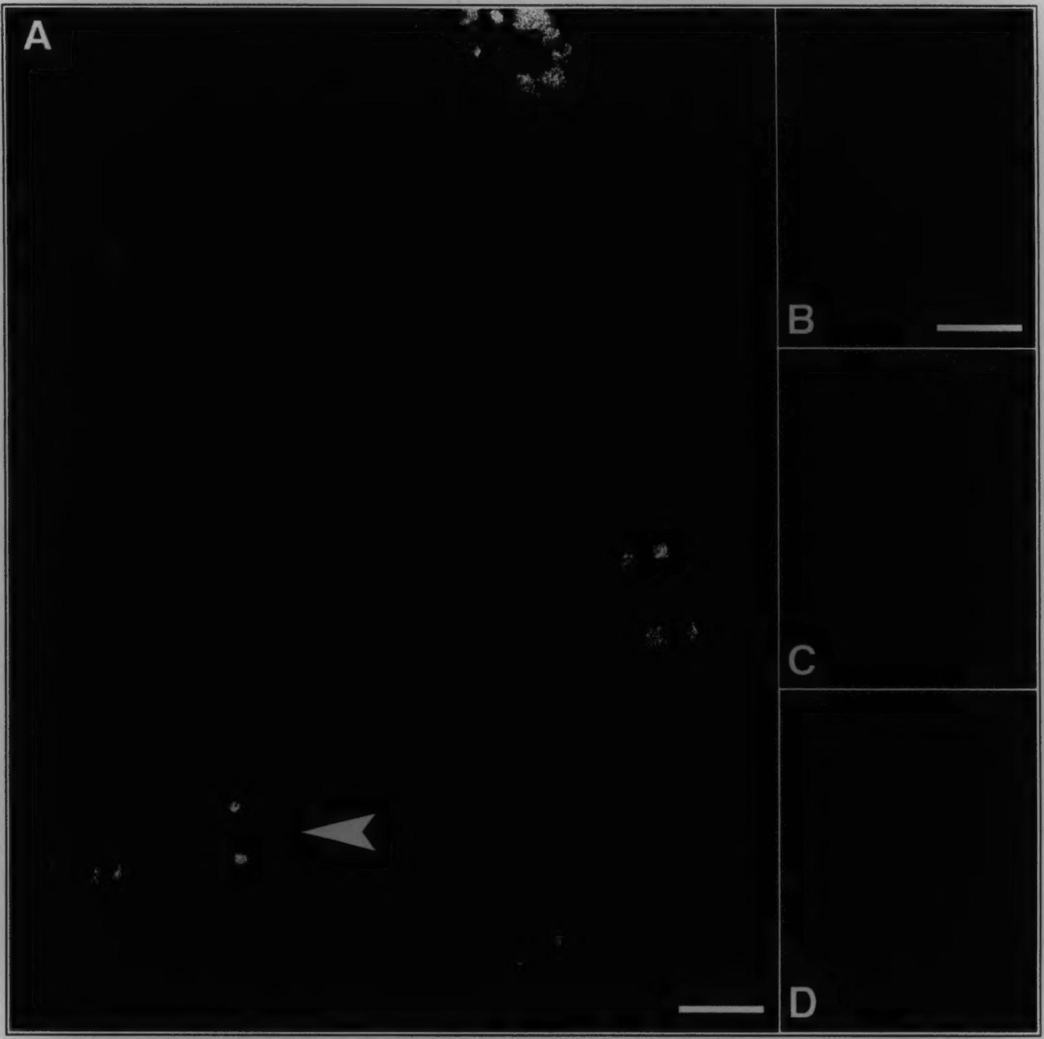

Figure 3.1 TAFII250, an acetyltransferase and part of the RNAPII holoenzyme required for HSV-1 gene expression, is recruited to viral compartments and is required for HSV-1 infection. Digital optical sections of SK-N-SH human neuroblastoma cells infected with HSV-1 and cultured for 6 hours post-infection. The cells were immunofluorescently costained with antibodies against the HSV-1 immediate early protein ICP4, present immediately at infection and recruited to viral replication compartments (H1101; C, red in panel A) and TAFII250 (D, green in panel A). Viral compartments are visible in the panel containing the chromatin stain DAPI, where the chromatin is absent in the nucleus (B, blue in panel D). The field of view in panel A contains uninfected cells without ICP4 staining and normal TAFII250 distribution patterns. Infected cells with established replication compartments show large ICP4 foci, concentration of TAFII250 in colocalized foci and exclusion of chromatin. The arrow indicates the position of a moderately infected cell enlarged for detail (inset panels B-D) showing recruitment. The scale bar in A represents 15 μm. The inset scale bar in B represents 5 μm. All transfected HAT species and antibodies against HATs from all acetyltransferase families tested showed recruitment of these proteins to viral compartments (Table 3.1).

cells show abnormal HAT distribution patterns, colocalizing with the HSV-1 immediate early protein ICP4 (red) in productive replication compartments. In Figure 3.2, the histone acetyltransferase CBP (red) shows recruitment to viral replication compartments *in situ*. This antibody against the C-terminus of CBP, which also recognizes PML bodies, is concentrated and colocalized with ICP4 (green) in established nuclear compartments. Moderately infected cells (left and middle) show a greater concentration of CBP in and around the viral compartment. CBP is grossly redistributed in the highly infected nucleus (right), probably correlating with the loss of PML bodies at later stages of infection. Again, nuclear compartments are clearly visible with DAPI chromatin staining (A). As with TAFII250, this enzyme is also involved in transcriptional initiation through chromatin remodeling and while it is responsible for a significant portion of histone acetyltransferase activities in the cell, it differs in that is not as a basal transcription factor and does not bind DNA.

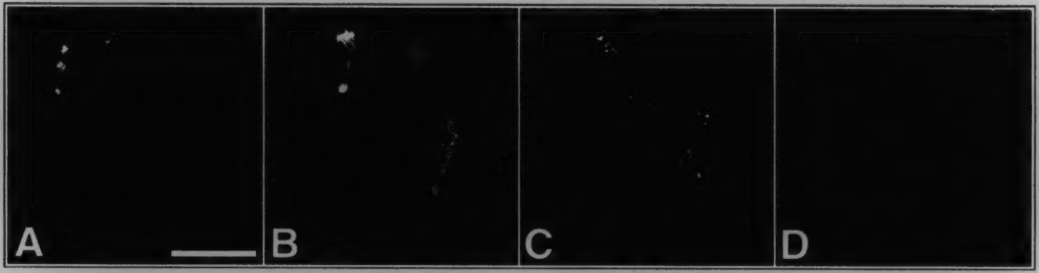

Figure 3.2 The histone acetyltransferase CBP is recruited to HSV-1 viral compartments. Digital optical sections were taken of SK-N-SH human neuroblastoma cells infected with HSV-1 and incubated for 6 hours before fixation. Viral compartments are visible in the panel containing the chromatin stain DAPI, where the chromatin is absent in the nucleus, as viral DNA is not packaged into chromatin (A, blue in panel D). The cells were immunofluorescently costained with antibodies against the HSV-1 immediate early protein ICP4 (H1101; B, green in panel D) and the C-terminus of the histone acetyltransferase CBP (C, red in panel D). The scale bar represents 15 μm. All transfected HAT species and antibodies against HATs from all acetyltransferase families tested showed recruitment of these proteins to viral compartments (Table 3.1).

Through indirect immunofluorescent analysis of transfected proteins, it was established that member proteins from all families of histone acetyltransferases are recruited to viral replication compartments in the nucleus. The data is summarized in Table 3.1. Endogenous CBP and p300, PCAF of the GNAT group, the TAFII250 basal transcription factor, members of the MYST family containing the characteristic MOZ motif, and the nuclear receptor cofactor ACTR all colocalized with the immediate early protein ICP4 of HSV-1 in established, productive viral replication compartments. Transiently transfected SK-N-SH, HISM and IM cells, as well as IM cells stably expressing CBP-GFP were also positive for recruitment. This was apparent in cells stained with Hoechst DNA dye to confirm the presence of viral compartments while fixed cells showed colocalization of the protein with the anti-ICP4 immunofluorescent staining. GFP alone, as a control, was abundant in the nucleus and cytoplasm but was not

Table 3.1 The recruitment of HATs and their potentially associated proteins to HSV-1 compartments.

Protein Recruited to HSV-1 Compartments	**Name**	**Endogenous**	**Transiently Transfected**[†]	**Stably Transfected**[†]
Histone Acetyltransferases	CBP	+	+[g]	+[g]
	p300	+	-	-
	ACTR	+	-	-
	PCAF	+	-	-
	MYST family	+	-	-
	TAFII250	+	-	-
Histone Acetyltransferase Potentially-Associated Proteins	AP-2	+	+[g]	+[g]
	CREB	+	-	-
	Estrogen Receptor	-	+[g]	+[g]
	p53	+	-	-
	Retinoblastoma	+	-	-

+ indicates that an assay for the particular protein was performed
- indicates that an assay was not performed for this protein
[†] g indicates GFP-protein constructs were used

recruited in transfected HeLa and IM cells (data not shown). Once HATs were shown *in situ* and *in vivo* to recruit to viral compartments, this confirmed that the HSV-1 model system was useful for characterizing functional nuclear organization and was used for further experimentation to determine what additional factors were recruited to viral compartments and the functional significance of this nuclear rearrangement.

In addition to the HATs recruited to HSV-1 replication compartments, proteins with known or hypothesized relationships with HATs are also found to be recruited to viral compartments. Positive results of these recruitment assays showing the typical enrichment of these proteins in viral compartments are found in Figure 3.3. The histone acetyltransferases CBP and p300 are coactivators of the pleiotropic transcription regulator Activator Protein 2 (AP-2), known to be involved with cAMP induction and implicated in the transcriptional control mechanisms of several viruses (Braganca *et al.*, 2003). Immunofluorescent staining using antibodies against AP-2 (C) show recruitment of the protein to viral compartments that are delineated by costaining with anti-ICP4 (B). This *in situ* recruitment of the endogenous protein population was also observed *in vivo* using AP-2-GFP transiently transfected in SKN and HISM cells and stably transfected in HeLa cells (Table 3.1). Viral compartments were visualized using Hoechst dye and infrared (IR) multi-photon spectroscopy to reduce phototoxic cell damage in live cells (data not shown). CBP and p300 are known to bind the transcription factor cyclic AMP response element binding protein (CREB) in its activated phosphorylated state (Eckner *et al.*, 1994). Figure 3.3 also shows the recruitment of this protein together with the cAMP responsive element modulator (CREM; bottom panels) transcription factor to HSV-1 compartments by immunofluorescently costaining with antibodies against an epitope recognizing both proteins and ICP4 (G and F respectively). Although both of these proteins are implicated in cell signaling, no direct interaction has been established for AP-2 and CREB/CREM.

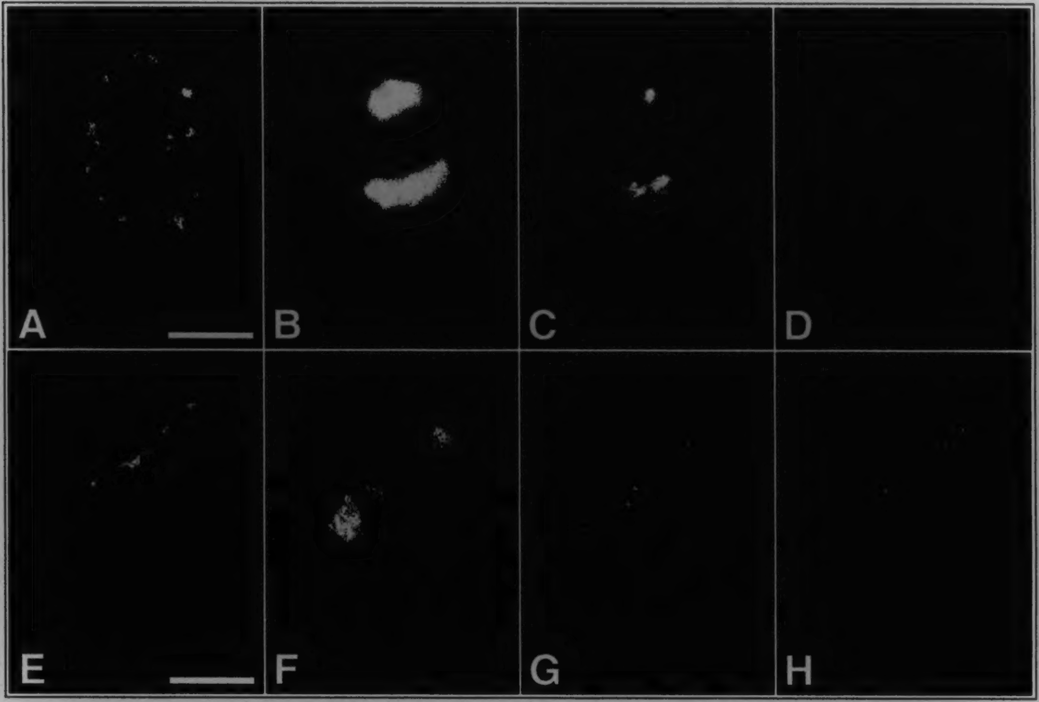

Figure 3.3 Proteins associated with histone acetyltransferases, but that are not known to have a direct relationship, are recruited to HSV-1 viral compartments. Digital optical sections were taken of an Indian Muntjack fibroblast cell (top) and an SK-N-SH human neuroblastoma cell (bottom) infected with HSV-1 and incubated for 6 hours before fixation. Viral compartments are visible in the panels containing the chromatin stain DAPI (A and E; blue in D and H). **Top:** The cells were immunofluorescently costained with antibodies against the HSV-1 immediate early protein ICP4 (B) to confirm the presence of viral compartments and against the Activator Protein 2 (AP-2; C). The composite image (D) shows a colocalization of ICP4 (green) and AP-2 (red) concentrated in areas occupied by viral compartments that are devoid of chromatin (blue). The scale bar in A represents 5 μm. **Bottom:** The cells were immunofluorescently costained with antibodies against the HSV-1 immediate early protein ICP4 (F) to confirm the presence of viral compartments and against the cAMP response element binding protein and modulator protein (CREB and CREM; G). The composite image (H) shows a colocalization of ICP4 (green) and CREB/CREM (red) concentrated in areas occupied by viral compartments that are devoid of chromatin (blue). The scale bar in panel E represents 5 μm. The antibodies to proteins associated with histone acetyltransferases that show recruitment are listed in Table 3.1.

The complete complement of proteins assayed for HSV-1 recruitment that are believed to be directly associated with histone acetyltransferases are listed in Table 3.1. Transcription regulated by estrogen involves interactions with the cointegrators CBP/p300 and PCAF but is only directly acetylated by p300 (Wang *et al.*, 2001). Transiently transfected HISM cells and stably transfected MCF-7 human breast cancer cells expressing estrogen receptor 1-GFP showed altered nuclear patterns and enrichment of the protein in large areas depleted of Hoechst DNA dye in live cells, demarking viral compartments, and costaining with ICP4 in fixed cells. The acetyltransferases PCAF and CBP/p300 are required for transcriptional activation of the tumor suppressor p53 and acetylation results in increased DNA binding of p53 (Gu *et al.*, 1997; Scolnick *et al.*, 1997). P53 tumor suppressor protein was described previously as being redistributed to HSV-1 compartments *in situ* (Wilcock and Lane, 1991; Zhong *et al.*, 2000). I corroborate these findings by reporting the recruitment of the activated phosphorylated serine-15 form of p53 *in situ*, a phosphorylated state believed to be integral to inducing a phosphorylation-acetylation cascade, with recruitment confirmed with costaining with ICP4 in SK-N-SH cells. The Retinoblastoma tumor suppressor protein (pRb) has recently been shown to be acetylated by p300 and this implicates acetylation in cell cycle progression (Chan *et al.*, 2001). Wilcock also describes the *in situ* recruitment of pRb to HSV-1 compartments (Wilcock and Lane, 1991), which is confirmed in SK-N-SH cells infected and costained with anti-pRb and anti-ICP4 antibody (see also Figure 3.16). It is at this point unclear whether sequestering of these proteins to HSV-1 replication compartments is a phenomenon related to their functional association with HATs. In this case, active or preferential recruitment of HATs to the viral replication compartment may result in recruitment through association by providing binding sites for associated proteins within the viral compartment. This seems more likely than the opposite case as HATs have a common activity in relationship to transcription while their associated proteins have various functions in a diverse range of transcriptional pathways. This does not completely exclude the possibility that some or all recruitment is a broader phenomenon brought about by the shut down of host transcription by the virus and the disruption of a functional nuclear organization.

3.2 Proteins within the Viral Compartment are not acetylated

The activity of histone acetyltransferases is specific entirely to the posttranslational reversible conversion of lysine functional groups on proteins to epsilon-acetyl lysines. However, because HSV-1 does not package its DNA into chromatin (Spencer *et al.*, 2000), acetyltransferases recruited to viral replication compartments cannot be acetylating nucleosomal lysines. Figure 3.4 shows the enrichment of CBP/p300 (B) *in situ* in the nuclear area occupied by the viral compartment, which is devoid of chromatin as seen using DAPI chromatin stain (A; denoted by v.c.). The presence of p300/CBP in viral compartments was confirmed by costaining with anti-ICP4 in previously described and subsequent recruitment experiments. Although CBP/p300 has been characterized as being able to hyperacetylate H2B and is part of chromatin remodeling and transcription complexes (Ito *et al.*, 2000), the viral compartment excludes this tetra-acetylated isoform of histone H2B (C). This is a general phenomenon observed for all acetylated histone species tested, both related to highly acetylated, dynamic class I histones and bulk acetylated class II histones. H2B continues to be acetylated in regions unoccupied by the viral compartment and in peripheral regions of the compartment, suggesting continued HAT activity during recruitment.

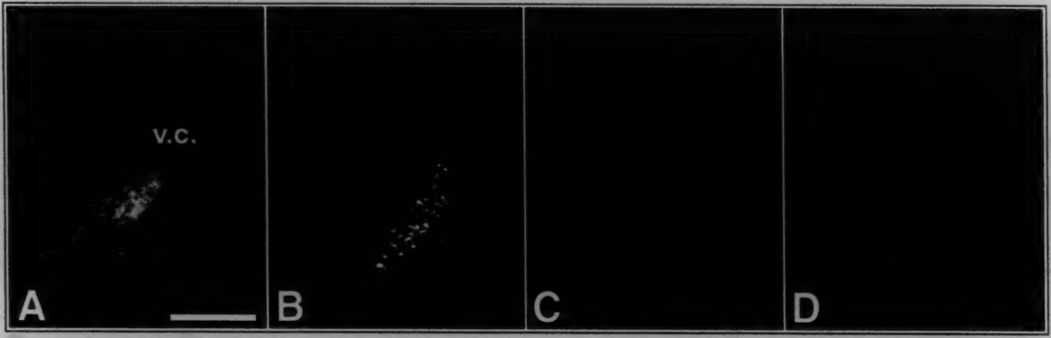

Figure 3.4 Acetylated histone species are absent in HSV-1 viral replication compartments that recruit histone acetyltransferases. Digital optical sections of an SK-N-SH neuroblastoma cell nucleus infected with HSV-1 and incubated for six hours prior to fixation. Viral replication compartments (v.c.) are clearly visible in the panel containing the chromatin stain DAPI, where the chromatin is absent in the nucleus (A). The cells were immunofluorescently costained with antibodies against the epitope common to the acetyltransferases CBP/p300 (B) and against the tetra-acetylated histone H2B isoform. The composite image shows the spatial distribution of CBP/p300 (green) in relation to the acetylated H2B isoform (red) and chromatin (blue). The scale bar represents 5 μm.

While chromatin and acetylated histone species are absent in viral replication compartments, this does not exclude the possibility, if acetyltransferase activity in recruited HATs is not deactivated, that factor acetyltransferase activity may be present in the viral compartment. I have demonstrated the recruitment of non-histone factors to viral compartments, as outlined above. Factor acetyltransferase activity has been implicated for a number of acetyltransferase-associated proteins recruited to viral compartments, including the tumor suppressor protein p53, the HAT ACTR, the cell cycle regulator pRb and the transcriptional coactivator CBP. These are potential substrates for CBP-specific factor acetyltransferase activity (Gu *et al.*, 1997; Chen *et al.*, 1999; Chan *et al.*, 2001; Lu *et al.*, 2003). The purpose of such acetylations hypothetically occurring within the viral compartment is unknown and speculative with respect to viral transcription or factor compartmentalization. To test this hypothesis of FAT activity, cells were infected and immunofluorescently costained with antibodies against ICP4 to label HSV-1 viral compartments and an antibody recognizing a variety of histone and non-histone acetylated lysine antigens. The result in Figure 3.5 shows that there are no discernable acetyl lysine residues (C) present within viral compartments (v.c.) whether they exist on histone or non-histone target proteins. This is consistent with all other immunofluorescent staining using acetylated histone antibodies on infected cells. Acetyltransferase activity does not appear to be occurring within the viral compartment on either histones or other factors. Interestingly, visible enrichment of acetyl lysine residues bordering viral compartments, as reported in Figure 3.4 with the acetylated H2B species, was commonly observed at all stages of infection. This is highlighted by red

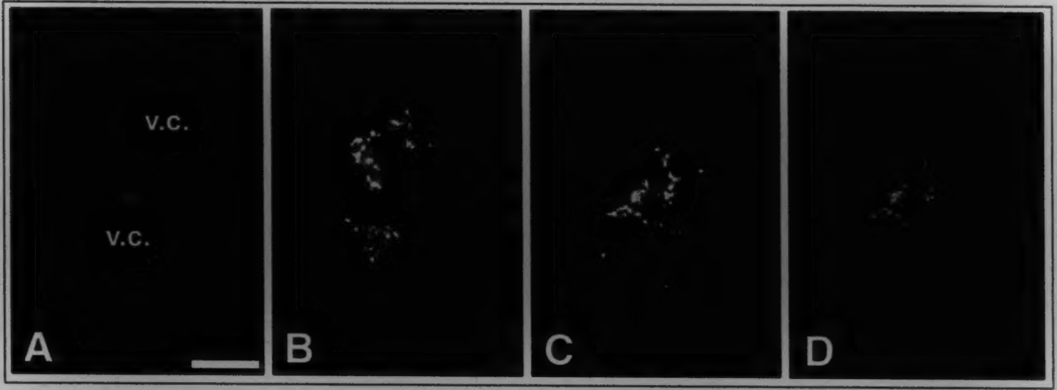

Figure 3.5 Histone acetyltransferases are recruited to viral compartments that are devoid of acetyl lysine residues. Digital optical sections of an Indian Muntjac fibroblast cell nucleus infected with HSV-1 and incubated for six hours prior to fixation. Viral compartments (v.c.) occupy the majority of the nuclear volume at this late stage of infection. They are clearly visible in the panel containing the chromatin stain DAPI (A). The cells were immunofluorescently costained with antibodies against the C-terminus of the histone acetyltransferase CBP (B) and acetyl lysine (C). The enzymatic activity of histone acetyltransferases is specific to the acetylation of lysine residues. Enrichment of acetyl lysine is consistently observed in the area surrounding viral compartments, as highlighted by red arrows (C). The composite image shows the spatial relationship between CBP (green) in the viral compartment, chromatin (blue) and acetyl lysine residues (red) occupying the space in between. The scale bar represents 3 μm.

arrows in panel C. This may suggest that, although acetyltransferase activity may not be required within the viral compartments, where the genome is not organized into chromatin, the enzymes remain catalytically active but depleted at steady-state outside of the replication compartment. An immunoblot using the acetyl lysine antibody confirmed that there are no discernable changes in the spectrum between infected and mock infected cells (data not shown).

3.3 RNA Polymerase II Shutdown Corresponds with Recruitment of Acetyltransferases to Viral Compartments

RNA Polymerase II is required for viral transcription and recruited to HSV-1 replication compartments before functional shutdown (Godowski and Knipe, 1986; Weinheimer and McKnight, 1987; Rice *et al.*, 1994; Jenkins and Spencer, 2001). RNAPII relies upon histone acetylation in normal host cells for chromatin modification required for transcriptional initiation (Tse *et al.*, 1998; Spencer and Davie, 1999; Eberharter and Becker, 2002). The concomitant recruitment of histone acetyltransferases to viral compartments may affect host RNAPII function. Figure 3.6 shows a reduction in RNAPII distribution through viral infection in a field of SK-N-SH neuroblastoma cells. A 5-fluoruridine pulse at a final concentration of 1 mM was added to the viral growth media five hours post-infection for fifteen minutes to label nascent RNA transcripts. The cells were then chased by incubating with fresh viral growth media one hour prior to fixation. To detect the halogenated nucleotide epitopes in RNA, the cells were immunofluorescently stained with anti-5-bromo-2'-deoxyuridine (BrdU; Sigma; green). Large arrows denote cells that are highly infected, characterized by large established viral

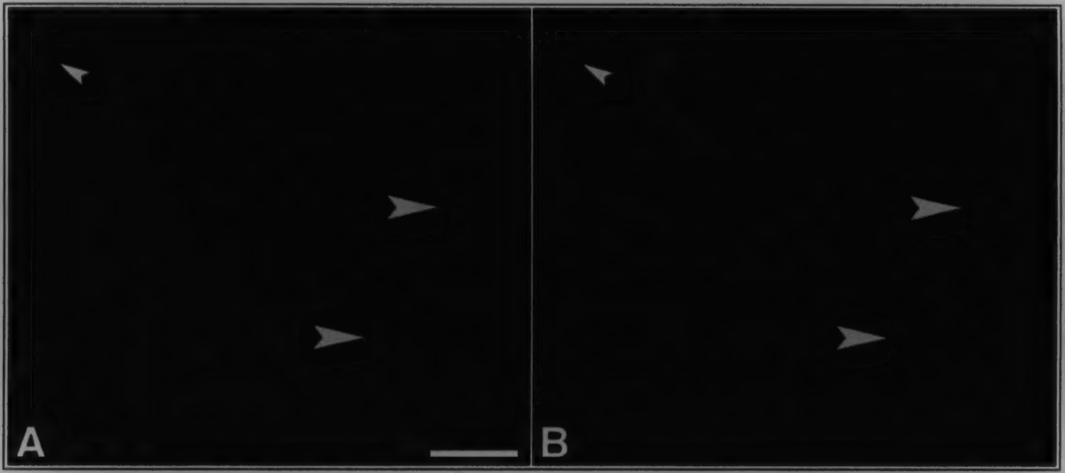

Figure 3.6 RNA Polymerase II activity is decreased with increased HSV-1 infection. A 5-fluorouridine pulse was added to the media of HSV-1 incubated SK-N-SH neuroblastoma cells for 15 minutes 5 hours post-infection and chased with fresh media for 60 minutes before fixation. Digital optical sections of a cell field show a decrease in RNAPII activity in highly infected cells, denoted by large arrows, stained with anti-BrdU to identify halogenated nucleotides in nascent RNA transcripts (A, green) and costained with ICP4 to show level of viral infection (red in B). The small arrow shows an indiscernible reduction in BrdU staining in a cell of low infection, showing an uncompartmentalized and diffuse ICP4 pattern, as compared to uninfected cells in the field. The scale bar indicates 15 μm.

replication compartments when costained with anti-ICP4 (red in B). These cells, where HATs are seen to be heavily concentrated within viral compartments as previously shown, display an overall decrease in RNAP activity, as seen by the decreased amount of 5-fluoruridine incorporated into RNA transcripts, compared to uninfected cells in the field. The small arrow denotes a cell with a low infection level, seen as a diffuse anti-ICP4 staining pattern, and no discernable decrease in transcription is observed. HATs are largely available to host chromatin as they are not heavily sequestered at this level of infection where viral prereplicative foci occupy a small volume of the nucleus.

Upon closer inspection, RNAPII activity is shut down in the host cell at a high level of infection where large viral compartments are established in the nucleus. Figure 3.7 shows the result of a pulse chase experiment on an SK-N-SH performed as described above. Viral compartments (v.c.) are easily discernable as areas devoid of chromatin in

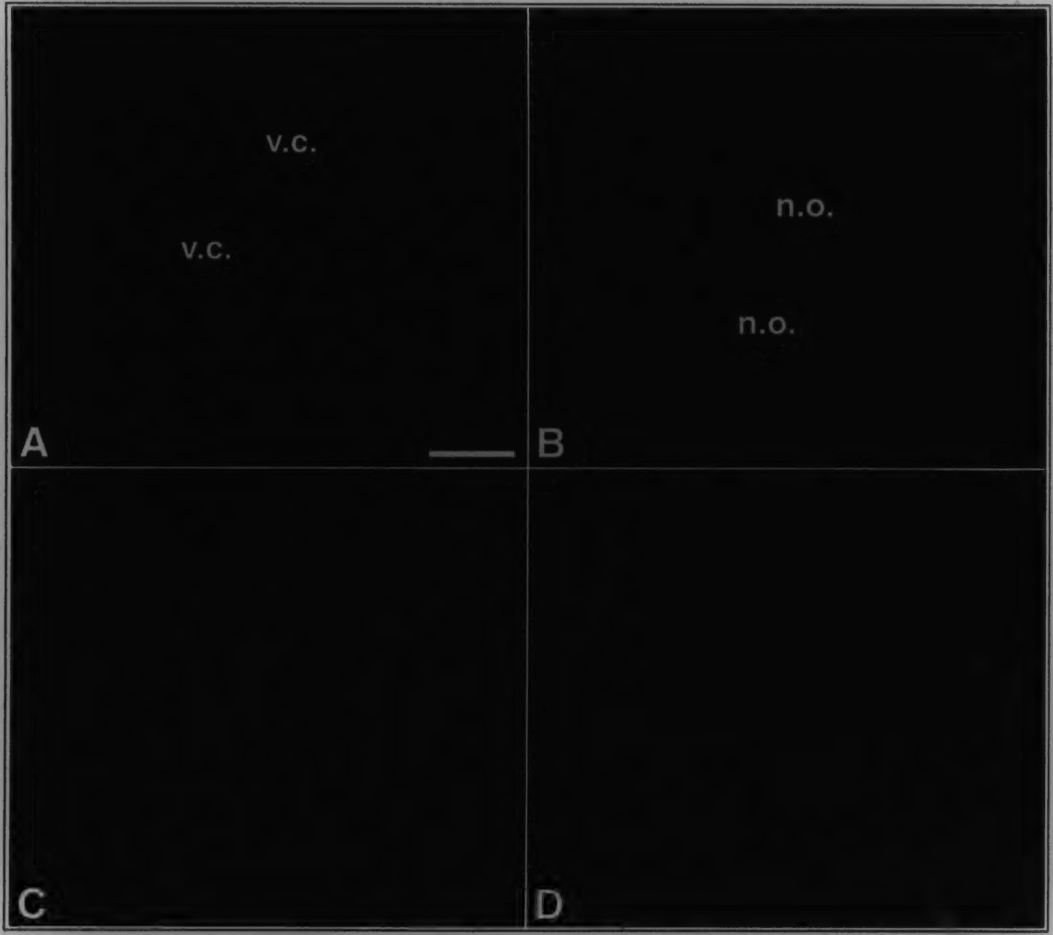

Figure 3.7 RNA Polymerase II activity is shutdown through HSV-1 infection. A 5-fluorouridine pulse was added to the media of HSV-1 incubated SK-N-SH neuroblastoma cells for 15 minutes 5 hours post-infection and chased with fresh media for 60 minutes before fixation. Digital optical sections show viral replication compartments (v.c.) are clearly visible as nuclear clearing in cells labeled with DAPI chromatin stain (A, blue). The cells were costained with anti-BrdU to label halogenated nucleotides in nascent RNA transcripts (B, green) and anti-acetylated H3 at K8 (C, red). The composite image (D) shows RNA transcripts are localized to the nucleolus (n.o.) but excluded from viral compartments, indicating RNAPII but not RNAPI activity is compromised. Scale bar represents 3 μm.

the nucleus when stained with DAPI (A, blue). Anti-BrdU labeling (B. green) reveals a heavy concentration of halogenated RNA transcripts in the nucleoli (n.o.) but is absent in viral compartments and is severely depleted or absent the perinuclear space. At this level of infection with large, established viral compartments, HATs are heavily concentrated and almost completely recruited within viral compartments, correlating with this loss of

RNAPII activity. This cell clearly retains an intact nucleolus at six hours post-infection, which it will lose in the final stages of the lytic cycle, and RNA polymerase I activity is not compromised at this infection level in contrast to RNAP II activity.

3.4 Histone Acetylation is decreased Through Viral Infection

Changes in steady-state histone acetylation levels could be involved in global chromatin condensation and host RNAPII transcriptional repression. I wished to determine whether or not the change in the steady-state distribution of HATs is reflected in a change in the steady-state levels of specific acetylated histone species.

To ascertain if there was any alteration in the acetylation of histones by HSV-1 on a global scale, isolated histones from infected and uninfected cell populations were run in 12% Acetic Acid Urea Triton X-100 (AUT) gels. Figure 3.8 is a typical result. At six hours post-infection, infected populations of Indian

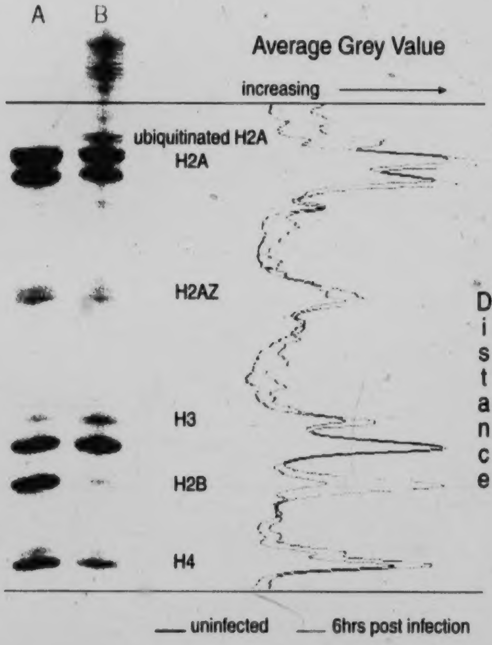

Figure 3.8 Histone acetylation patterns are altered during HSV-1 infection. Indian Muntjack fibroblast cell histones from populations uninfected and infected for 6 hours (A and B respectively) were run on an AUT gel. The average grey value of each lane is charted versus the distance migrated for each histone isotope group. Although there appears to be subtle differences in some acetylation isoforms for core histones between uninfected (green) and infected (red) cells, the AUT gel is not adequately resolving to draw conclusions regarding populations of specific acetylation states during infection.

Muntjac fibroblast cells show high infection levels, there is significant recruitment of HATs to HSV-1 compartments and a demonstrated severe reduction in RNAPII activity. Upon visual inspection and data analysis of the pixel intensity of the protein bands stained with Coomassie Brilliant Blue, little discernable difference in acetylation patterns is observed. There seems to be a slight increase in H3 acetylation and decreases in H2A, H2B and H4. Interestingly, there seems to be increased ubiquitination of histone H2A, which is implicated in a large number of biological processes (Cheung *et al.*, 2000). However, this method would normally resolve the various acetylated isotopes for each histone species. I ascertained that AUT gels are not adequately resolving so as to quantitate the differences between isoforms and draw specific conclusions as to the nature of acetylation between these two states at these times of infection. Consequently, I performed *in situ* immunofluorescent assays and *in vitro* immunoblotting using several antibodies to test the level of acetylation for specific acetylated histone species through HSV-1 infection.

Figure 3.9 illustrates the decrease of highly acetylated histone H2B species from low infected to highly infected Indian Muntjac fibroblast cells. Indirect immunofluorescent images were taken six hours post infection and stained with the chromatin stain DAPI (A), antibodies against the HSV-1 immediate early protein ICP4 to identify viral compartments and indicate the level of infection (B) and anti-tetra acetylated histone H2B (C). It is important to again note the distinction made between what are called levels of infection, based upon viral compartment morphology and chromatin structure, and that of the expression of viral proteins through the immediate-early, early and late genes, which are not correlated with compartment size and

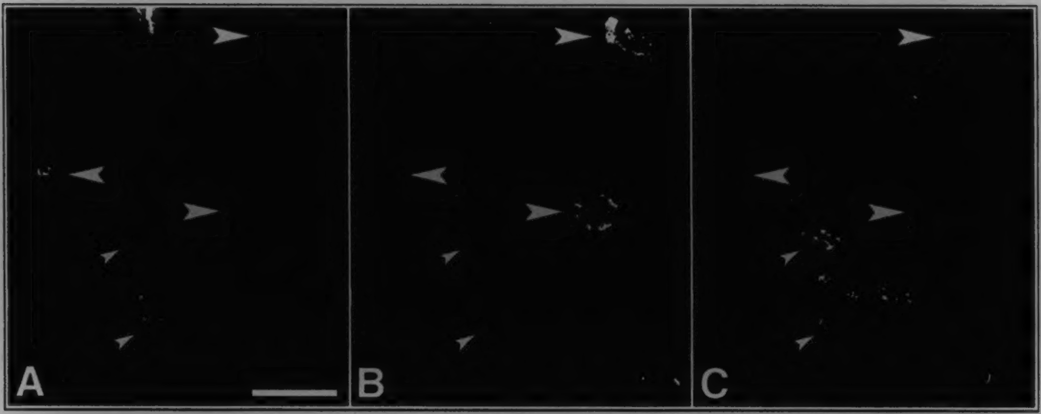

Figure 3.9 Histone acetylation is visibly decreased through HSV-1 infection. Digital optical sections of Indian Muntjac fibroblast cells infected and incubated for six hours before fixation. DAPI chromatin stain allows for the identification of nuclei (A). The cells were stained with antibody against the immediate early viral protein ICP4 (B) as an indication of the level of infection. Large and small arrowheads indicate highly infected and low infected cells respectively in all panels. Antibody recognizing all acetylated epitopes on histone H2B (C) was used to measure acetylation relative to chromatin content. Immunofluorescent data analysis for acetylation assays is shown in Table 3.2. The scale bar represents 20 μm.

morphology (see Materials and Methods). Low infected cells, indicated by small angled arrows, show a diffuse anti-ICP4 staining pattern with several small punctate prereplicative sites and little deviation from normal chromatin DAPI staining patterns. Highly infected cells, indicated by large arrows, have few well-established annexed replication compartments, with large nuclear volume occupancy depleted of chromatin and enriched in ICP4. Compared to the low infected cells, these cells display a marked visible decrease in AcH2B signal intensity (C) indicating a decrease in this acetylation. Additionally, the hyperacetylated isoforms of H2B are implicated in transcriptionally active chromatin. Therefore, a decrease in AcH2B through infection also implies the loss of a transcriptionally relevant acetylation.

It is reasonable to suggest that viral recruitment of HATs may be responsible for a decrease in histone acetylation though altering the steady-state of HAT populations. To refine and expand upon visual immunofluorescence observations, an *in situ* assay for

Table 3.2 *In situ* assay for acetylation for specific acetyl lysine epitopes in Indian Muntjack fibroblast cells.

Acetylated Lysine Residues	Infection Level	n	Mean Acetylation/ DAPI +/-SD	p-value	Statistical Significance
Penta-acetylated H4	Low	78	1.000 +/- 0.294	n/a	
	Medium	111	0.735 +/- 0.255	p<0.0001	+++
	High	209	0.662 +/- 0.217	p<0.0001	+++
K9 and K14 of H3	Low	175	1.000 +/- 0.561	n/a	
	Medium	57	0.767 +/- 0.313	p=0.0031	++
	High	30	0.768 +/- 0.347	p=0.0295	+
Total H2B	Low	175	1.000 +/- 0.658	n/a	
	Medium	76	0.831 +/- 0.458	p=0.0412	+
	High	34	0.737 +/- 0.258	p=0.0215	+
K8 of H4	Low	127	1.000 +/- 0.578	n/a	
	Medium	65	1.046 +/- 0.393	p=0.5468	-
	High	68	1.055 +/- 0.437	p=0.4933	-

relative acetylation of chromatin was designed to quantify digital confocal image data and assess whether or not histone deacetylation is a global phenomenon or more specific to dynamic or basal acetylation levels due to recruitment. The antibodies used were anti-penta acetylated H4, anti-acetylated H3 specific for lysines 9 and 14, hyperacetylated H2B (as in Figure 3.8) and anti-acetylated H4 specific for acetyl lysine 8. The data was normalized relative to low infection stages at 1.0 for ease of comparison and the results are summarized in Table 3.2. There is a significant loss in acetylation between low infected hyperacetylated H4 species and

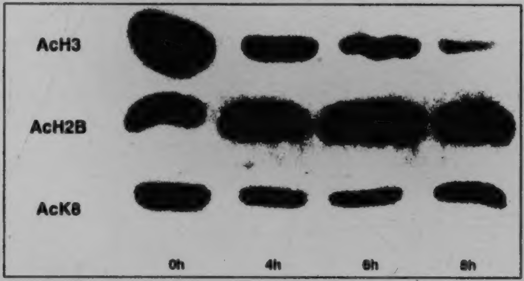

Figure 3.10 Transcriptionally associated histone acetylation is significantly lost through HSV-1 infection. The top immunoblot shows an incremental decrease in anti-acetylated H3 acetyl K9 and the transcriptionally relevant acetyl K14 relative to uninfected control cells. Bulk acetylation levels, represented in the lower panel by acetyl lysine 8 of H4, show a decrease in acetylation in all time points over the control but relatively little changes through infection. There is no visible difference between control and infected cells for AcH2B.

their medium and highly infected counterparts ($p<0.0001$ for both populations). H3 acetylation also shows a decrease from the low infected status through infection. Hyperacetylation of H2B, a result of dynamic acetylation and important to nucleosome alteration but to a lesser extent transcription (Ito *et al.*, 2000; Morales and Richard-Foy, 2000), is slightly but significantly reduced through infection. There is no loss in overall bulk class II acetylation as K8 of H4 acetylation is not decreased through medium and high infection levels. Immunoblotting confirmed the *in situ* results in that acetylated histone H3 was significantly and incrementally lost through infection time as compared to control uninfected cells while there was not a significant decrease in acetyl lysine 8 of histone H4 during the course of viral infection (Figure 3.10). As well, hyperacetylated H2B species were not discernibly lost through these infection times, which do not support the *in situ* data. A possible explanation for the trend of significant losses in class I acetylation-related acetyl lysine epitopes may be preferential recruitment of certain HATs to viral compartments causing a loss of a preferential site acetylation or one without significant site acetylation overlap.

3.5 Histone Acetyltransferase Activity in Infected Cells

The loss of specific dynamic class I associated lysine acetylations important to chromatin remodeling in transcription appeared to be less likely the result of inactivation of HATs than of increased activity of HDACs. This is partially evidenced by the presence of enriched acetylated lysine epitopes present on the periphery of viral compartments where HATs are recruited, as indicated by arrows in Figure 3.5C. Rather, the preferential recruitment of HATs to viral compartments may alter the steady-state activity to favor net deacetylation.

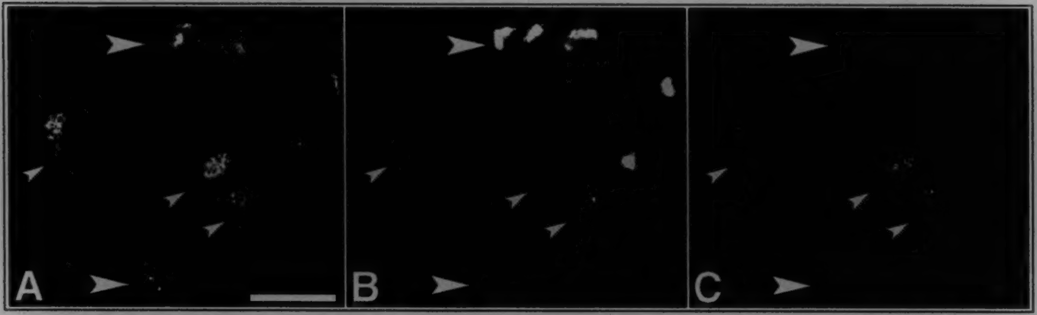

Figure 3.11 Histone acetyltransferase activities are decreased through HSV-1 infection, as detected by a loss of highly acetylated histone species. Digital optical images of SK-N-SH neuroblastoma cells infected with HSV-1 and cultured for four hours were taken. Cells were treated with 100 nM Trichostatin A before fixation. DAPI chromatin stain allows for comparison of chromatin content and the identification of nuclei (A). The cells were fixed and costained with antibody against the immediate early viral protein ICP4 (H1101; B) as an indication of the level of infection and antibody recognizing highly acetylated species of histone H3 (AcH3; C) was used to measure acetylation relative to chromatin content. Large arrowheads indicate the positions of highly infected cells with well-established viral replication compartments. Small arrowheads indicate the positions of infected cells with very small or no visible replication compartments. Immunofluorescent data analysis for acetyltransferase activity assays are shown in Table 3.3. The scale bar represents 20 μm.

To assess whether or not histone specific HAT activity is compromised through HSV-1 infection, an *in situ* HAT assay was performed. Consistent with *in situ* HAT assay data, upon visual inspection, Figure 3.11 illustrates that less hyperacetylation is observed during treatment with the HDAC inhibitor Trichostatin A (TSA) in later stages of viral infection. As with cells involved in the *in situ* HAT assay, indirect immunofluorescent images of SK-N-SH infected for four hours and treated with 100 nM TSA one hour prior to fixation were taken. Cells were stained with the chromatin stain DAPI (A) to allow for comparison of chromatin content and the identification of nuclei. Antibodies against the HSV-1 immediate early protein ICP4 were used to identify viral compartments and indicate the level of infection (B) and anti-H3 acetylated in lysines 9 and 14 (C) was used to ascertain the level of transcriptionally relevant dynamic acetylation relative to chromatin content. Large arrowheads indicate the positions of highly infected cells with well-established viral replication compartments. These clearly show a loss of hyperacetylated species compared to cells with very small or no visible replication compartments whose positions are denoted by small arrowheads.

In situ HAT assay data, extracted from confocal images of infected Indian Muntjac fibroblast cells treated with 100 nM TSA 1 hour before fixation, is presented in Table 3.3. In this assay, hyperacetylation is consistently observed at early stages of infection with all antibodies tested. As such, if the histone acetyltransferase activities responsible for a particular acetylation are reduced or HDACs are activated during the course of infection, there will be a decrease in the relative acetylation state of cells imaged at later stages of infection when compared to early stages, which are normalized to 1.0 for ease of comparison. Some changes in the relative HAT activities were evident. A net increase in H3 HAT activity in the middle stages of infection is observed, but this

Table 3.3 *In situ* assay for HAT activity detected for specific acetyl lysine epitopes in Indian Muntjack fibroblast cells.

Acetylated Lysine Residues	Infection Level	n	Mean Acetylation/ DAPI +/-SD	p-value	Statistical Significance
Penta-acetylated H4	Low	88	1.000 +/- 0.484	n/a	
	Medium	133	0.698 +/- 0.275	p<0.0001	+++
	High	113	0.573 +/- 0.254	p<0.0001	+++
K9 and K14 of H3	Low	116	1.000 +/- 0.493	n/a	
	Medium	49	1.005 +/- 0.377	p=0.9494	-
	High	101	0.724 +/- 0.304	p<0.0001	+++
Total H2B	Low	242	1.000 +/- 0.740	n/a	
	Medium	92	0.747 +/- 0.885	p=0.0087	++
	High	47	0.317 +/- 0.168	p<0.0001	+++
K8 of H4	Low	213	1.000 +/- 0.580	n/a	
	Medium	75	0.912 +/- 0.508	p=0.2447	-
	High	60	0.958 +/- 0.972	p=6751	-

returns to the levels of the low infection level at later stages of infection. There is also a very significant net decrease in HAT activity detected for penta-acetylated H4 epitopes ($p<0.0001$) and also for hyperacetylated H2B. Despite the slight variation in relative HAT activities, there exists a trend towards a decrease in HAT activity on highly acetylated transcriptionally related epitopes through infection but this does not include the moderately acetylated state having acetylated lysine 8 on H4. Therefore, HATs remain active through to late stages of infection, supporting image data showing enriched acetyl lysine populations on chromatin adjacent to viral compartments (Figure 3.5C). Through this assay it is determined that HATs participate less in concentrated class I dynamic acetylation as infection progresses.

3.6 Histone Deacetylases and Potentially Associated Proteins are recruited to Viral Compartments

Histone acetylation levels are dynamic and governed by the net activities of HATs and HDACs. Several possible scenarios may be drawn to explain the loss of highly acetylated histone species. These include: (1) If histone deacetylases are minimally or not recruited to viral compartments, HDACs would maintain a similar distribution pattern as non-infected cells and the antagonistic steady-state activity between HATs and HDACs would be shifted to favor deacetylation; (2) HDACs become more activated, and, along with HAT recruitment, deacetylation is again favored (3) The enzymatic activity of HATs is decreased or deactivated early in infection, prior to complete recruitment; (4) There is preferential recruitment of HATs over HDACs and, with the differential timing, a shift favorable to HDAC activity is still possible.

To address primarily the first scenario, recruitment assays were performed to ascertain if HDACs were recruited to viral compartments *in situ*. Antibodies against endogenous HDACs, HDAC-GFP fusion proteins and FLAG-tagged constructs were used. Both class I RPD3-like proteins and class II HDA1-like proteins were recruited to HSV-1 compartments, specifically HDAC1, 2, and 3 from the former class and HDAC4, 5 and 7 from the latter (Table 3.4). The top row of Figure 3.12 illustrates the recruitment of HDAC1 to HSV-1 compartments. These panels show examples of both early-infected or non-infected and late infected SK-N-SH neuroblastoma cells, with the large viral compartments, denoted by v.c. in the DAPI chromatin image (A) and the HDAC1 antibody staining pattern in panel B. The bright focus in panel B near the middle of the left nucleus is not a typical focus for HDAC1 staining (Hendzel *et al.*, 1998) and may indicate a low level of infection. Panel C is a composite image of chromatin (blue) and HDAC1 (red) and demonstrates that, while HDAC1 remains in the interchromatin space in both highly and low infected cells, HDAC1 is enriched in the viral compartments and depleted in the remaining interchromatin space. I confirmed that this was not simply preferential fixation of HDAC1 in viral compartments. SK-N-SH, IM, COS and HISM cells with transfected HDAC1-GFP recruited the protein construct to viral compartments in both living and fixed cells (Table 3.4). It is of interest to note that this HDAC1-GFP does not localize properly under normal uninfected conditions and may indicate a more general recruitment phenomenon of proteins through infection (M. Hendzel, personal communication). This recruitment of HDAC expressing cells is not limited to HDAC1. Panel D of Figure 3.12 shows a clearing in the chromatin stain DAPI in the nucleus of an Indian Muntjac fibroblast cell indicative of a large viral compartment (v.c). HDAC3-

FLAG is enriched in this area devoid of chromatin in a manner inconsistent with nuclear localization in mock infected cells (Emiliani *et al.*, 1998). Panel F is a composite image of DAPI (blue) and HDAC3-FLAG (red) and demonstrates a distinct enrichment of HDAC3 within the viral compartment.

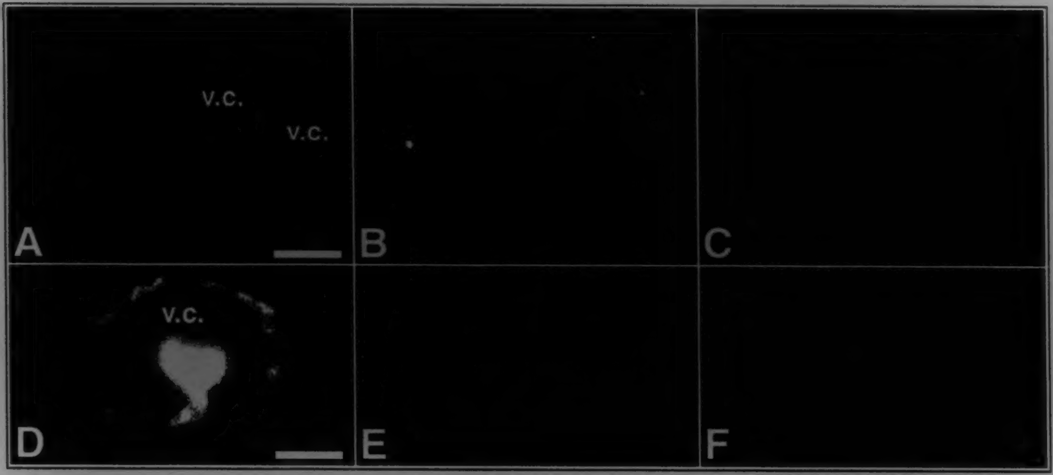

Figure 3.12 Class I histone deacetylases are recruited to HSV-1 compartments. **Top:** Digital optical sections of SK-N-SH neuroblastoma cells infected and incubated for six hours prior to fixation. The cells were labeled with the chromatin stain DAPI (A). Viral DNA is not packaged into chromatin and is observed as a clearing in the nucleus, denoted as v.c. Cells were immunofluorescently stained with anti-HDAC1 (B). The composite image in panel C shows the redistribution of HDAC1 and chromatin in the infected cell versus the low or non-infected cell in the field of view, where viral compartments are not established. The scale bar in panel A represents 5 µm. **Bottom:** Indian Muntjac fibroblast cells were transiently transfected with HDAC3-FLAG tag construct 24 hours prior to infection. Digital optical sections were taken of cells infected and incubated six hours prior to fixation. Viral compartments (v.c) are visible as clearing in the chromatin stain DAPI (D). Immunofluorescent staining with anti-FLAG (E) revealed abnormal distribution of the expressed protein. The composite image (F) shows recruitment of HDAC3 (red) in viral compartment areas devoid of chromatin (blue). The scale bar in panel D represents 5 µm. All transfected class I HDAC species and antibodies against class I HDACs tested showed recruitment of these proteins to viral compartments (Table 3.4).

Class II HDACs were discovered more recently and, like their class I counterparts, are able to deacetylate acetyl lysine groups, form large multiprotein complexes, some containing HDAC3, and play a role in differentiation and proliferation (Wang *et al.*, 1999; Fischle *et al.*, 2001). Normal distribution of HDAC4, 5 and 7 in mock-infected cells shows primarily nuclear and distinct subnuclear localization as well as a smaller cytoplasmic population. This cellular localization is suggested to be contingent upon function (Fischle *et al.*, 1999; Grozinger and Schreiber, 2000; Fischle *et al.*, 2001). Figure 3.13 shows the nuclear population of endogenous HDAC4 (C, red in D) in SK-N-SH cells enriching in viral compartments and colocalizing with the HSV-1 immediate early protein marker for infection, ICP4 (B, green in D), which are both excluded from heterochromatin regions (A). The bottom panels of Figure 3.14 show an Indian Muntjac fibroblast cell transfected with a FLAG tagged HDAC4 232 fragment, implicated in nuclear targeting and truncated to exclude the functional domain. After HSV-1 infection and incubation for six hours, the result is a nucleus with a large viral compartment, denoted by v.c., observed when stained with DAPI (E). The immunofluorescent staining pattern of anti-FLAG tag (F) shows abnormal enrichment of the HDAC4 fragment similar to the endogenous protein in C and different from mock infected cells. The composite image in panel G shows that enrichment of the truncated HDAC4 (red) is localized in the viral compartment that is devoid of chromatin (blue). This may indicate that recruitment of HDACs to viral compartments is indiscriminant of the HDAC function. Recruitment may involve a binding site on the small 232 amino acid region. However, it may hint at recruitment being a more global phenomenon rather than sequence or function dependent. The same recruitment to viral compartments was observed for endogenous HDAC5 and 7 and transiently expressing HDAC4-FLAG SK-N-SH, IM and HISM cells (Table 3.4).Not only were HDACs from both classes recruited to viral compartments but all proteins tested with suggested associations with HDACs were also recruited to viral compartments (Table 3.4). mSin3A is a transcriptional corepressor whose action of global dynamic deacetylation of chromatin is mediated by a

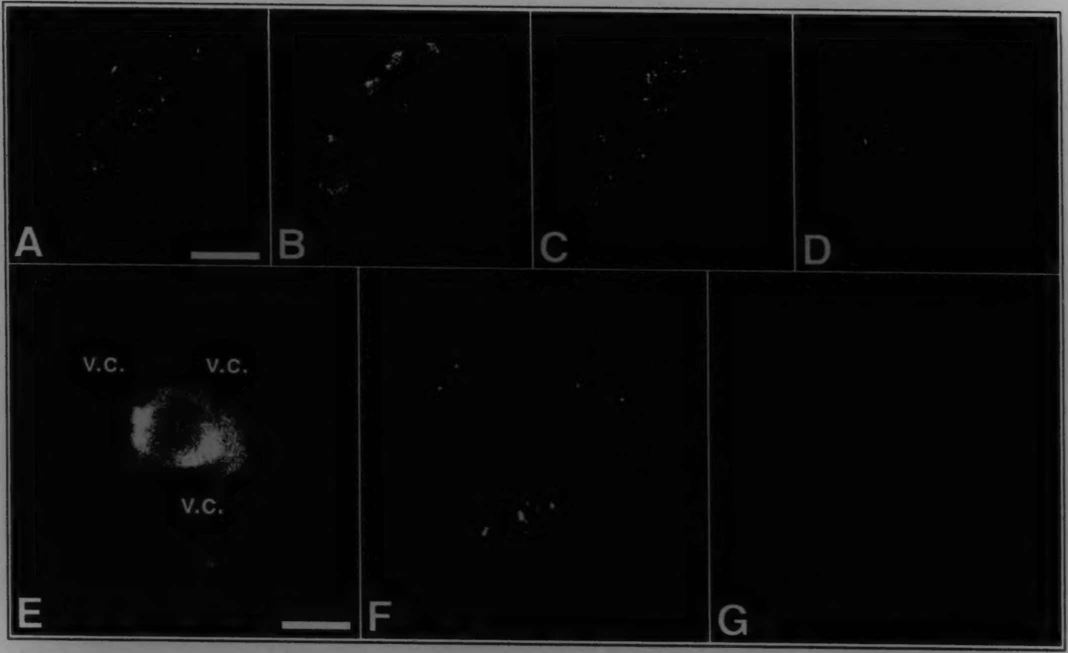

Figure 3.13 Class II histone deacetylases are recruited to HSV-1 compartments. Digital optical sections of Indian Muntjack fibroblast cells infected with HSV-1 and incubated for six hours prior to fixation. **Top:** Cells were stained with the chromatin stain DAPI (A) and immunofluorescently costained with an antibody against the immediate early HSV-1 protein ICP4 (B) and anti-HDAC4, a class II histone deacetylase (C). The composite image in panel D shows the enrichment of HDAC4 (red) in viral compartments, shown by ICP4 (green), and the depletion of the protein in interchromatin areas (blue). The scale bar in panel A represents 10 µm. **Bottom:** SK-N-SH neuroblastoma cells were transiently transfected with an HDAC4-FLAG tag construct that only expresses the nuclear localization fragment 24 hours prior to infection. Digital optical sections were taken of cells infected and incubated 6 hours prior to fixation. Viral compartments (v.c) are visible as clearing in the chromatin stain DAPI (E). Immunofluorescent staining with anti-FLAG (E) revealed abnormal enrichment of the expressed protein in the viral compartment, similar to that of the endogenous HDAC4 protein in panel C. The composite image (G) shows recruitment of the HDAC4 fragment (red) in viral compartment areas devoid of chromatin (blue). The scale bar in panel E represents 3 µm. All transfected class II HDAC species and antibodies against class II HDACs tested showed recruitment of these proteins to viral compartments (Table 3.4).

direct association with the class I histone deacetylases HDAC1 and HDAC2 within Sin3 corepressor complexes (Struhl, 1998; Zhang *et al.*, 1998; Rosenfeld and Glass, 2001; Li *et al.*, 2002). Topoisomerase II is a ubiquitous protein involved in many cellular processes including transcriptional control, apoptosis, DNA replication and repair and chromosome segregation. It is shown to associate directly *in vivo* with class I HDACs 1 and 2 as a component of the NuRD but not Sin3 corepressor complex. Therefore no direct relationship has been established between topoisomerase II and HDAC1 and HDAC2. Whether this association of Topoisomerase II with basal transcription factors leads to differential gene expression has not completely been elucidated (Tsai *et al.*, 2000; Collins *et al.*, 2001; Johnson *et al.*, 2001). Figure 3.14 shows the recruitment of the HDAC associated proteins through immunofluorescent costaining of mSin3A (C) and Topoisomerase II (G) with the immediate early HSV-1 protein ICP4 (B and F) in

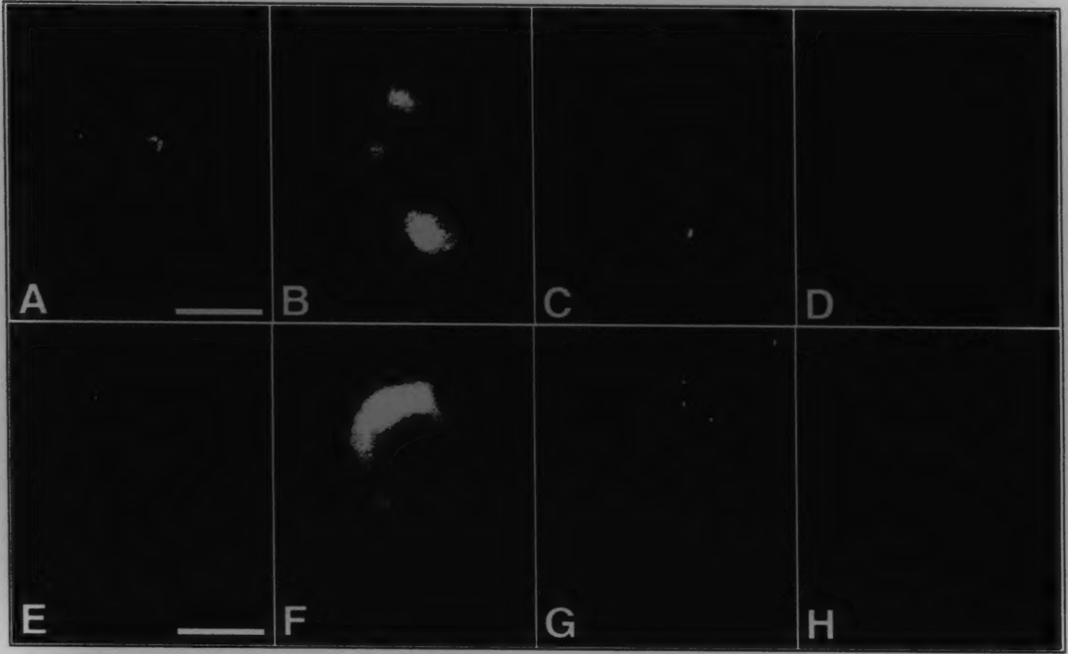

Figure 3.14 Proteins associated with histone deacetylases are recruited to HSV-1 viral compartments. Digital optical sections were taken of SK-N-SH cells (top) and Indian Muntjac fibroblast cells (bottom) infected with HSV-1 and incubated for six hours. Viral compartments are visible in the panels containing the chromatin stain DAPI (A and E). **Top:** The cells were immunofluorescently costained with antibodies against the HSV-1 immediate early protein ICP4 (B) to confirm the presence of viral compartments and against MSin3A (C). The composite image (D) shows a colocalization of ICP4 (green) and MSin3A (red) concentrated in areas occupied by viral compartments that are devoid of chromatin (blue). The scale bar in panel A represents 5 µm. **Bottom:** The cells were immunofluorescently costained with antibodies against the HSV-1 immediate early protein ICP4 (F) to confirm the presence of viral compartments and against Topoisomerase II (G). The composite image (H) shows a colocalization of ICP4 (green) and Topoisomerase II (red) concentrated in areas occupied by viral compartments that are devoid of chromatin (blue). The scale bar in panel E represents 10 µm. All transfected species and antibodies against proteins associated with histone deacetylases tested showed recruitment of these proteins to viral compartments (Table 3.4).

SK-N-SH neuroblastoma cells and Indian Muntjac fibroblast cells respectively. Recently, there is extensive evidence to support Topoisomerase II in being implicated in viral DNA synthesis and the transcription of late genes *in vitro* (Advani *et al.*, 2003) but this is the first report of association of the protein within viral compartments *in situ*. The only additional protein that was positive for HSV-1 recruitment with a known association only to HDAC1 is Ataxia-Telangiectasia mutated (ATM), which is implicated in cell cycle checkpoint control and double strand break repair (Kim *et al.*, 1999). Interestingly, double strand break repair may occur on HSV-1 DNA genome ends at PML bodies (Maul *et al.*, 1996) but ATM has not been implicated.

Retinoblastoma protein is classically described as a transcriptional repressor involved in the recruitment of class I HDAC1 and HDAC2 activities in transcriptional repression of E2F promoters to ensure proper cell cycle progression (Luo *et al.*, 1998). Recently a potential indirect association has also been reported with class II HDACs (Wade, 2001). Figure 3.15 shows the results of a recruitment assay for the protein in HSV-1 infected SK-N-SH neuroblastoma cells. Immunofluorescent costaining of cells infected and incubated six hours prior to fixation (A) yielded both infected cells, those labeled with anti-ICP4 immediate early protein (red), and uninfected cells without red label showing normal punctate pRb distribution (green) in the interchromatin space surrounding heterochromatin (blue). Infected cells with large, established nuclear compartments (C) show the typical exclusion of chromatin (B) and an altered nuclear distribution and enrichment of pRb in this area (D). However, as aforementioned, pRb may not only be a component of multimeric HDAC complexes but are sequestered into multimeric HAT complexes implicated in the transformation of cells by the E1A

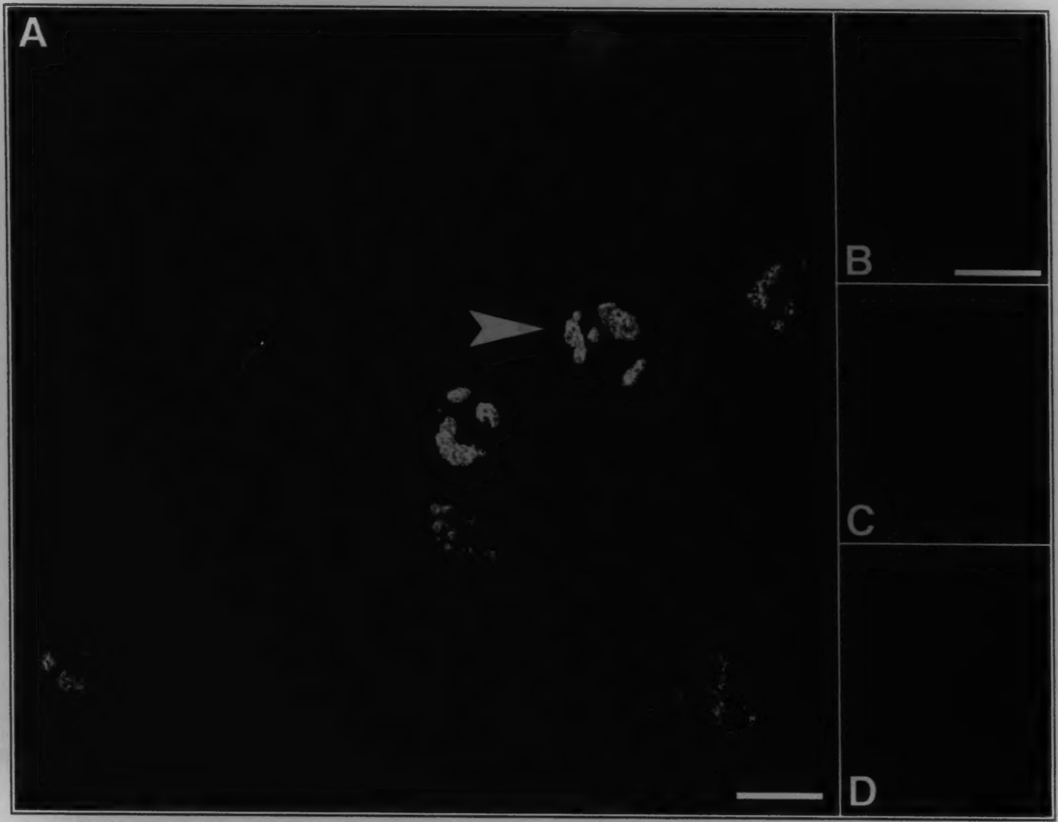

Figure 3.15 The retinoblastoma protein, associated in both multimeric HAT and HDAC complexes, is recruited to viral compartments. Digital optical sections of SK-N-SH human neuroblastoma cells infected with HSV-1 and cultured for six hours post-infection were taken. The cells were immunofluorescently costained with antibodies against the HSV-1 immediate early protein ICP4 to indicate the level of infection and confirm the presence of viral compartments (C, red in panel A) and anti-Retinoblastoma protein (D, green in panel A). Viral compartments are visible in the panel containing the chromatin stain DAPI, where the chromatin is absent in the nucleus (B, blue in panel D). The field of view in panel A contains uninfected cells without ICP4 staining and normal pRb distribution patterns. Infected cells with established replication compartments show large ICP4 foci, concentration of pRb in colocalized foci and exclusion of chromatin. The arrow indicates the position of a moderately infected cell enlarged for detail (inset panels B-D) showing recruitment. The scale bar in A represents 15 μm. The inset scale bar in B represents 5 μm.

adenoviral oncoprotein to bypass cell cycle control (Chan *et al.*, 2001). Additionally, the remaining HSV-1 recruited proteins listed in Table 3.4 have multifunctional roles as they have recently been associated with both HAT activities, as described previously, and HDAC activities. Estrogen receptor, under a histone deacetylase-dependent transcriptional control mechanism, may form a complex with HDAC3 and HDAC6, class I and II histone deacetylases respectively, mediated by an interaction with Ligand-dependent Nuclear Corepressor Receptor (LiCoR; (Fernandes *et al.*, 2003). It is the only HDAC-associated protein assayed for viral recruitment with a known direct interaction with class II deacetylases. As p53 is acetylated to stabilize and transcriptionally activate the protein in response to cellular stress, HDAC1 has been shown to indirectly bind a key negative regulator of p53, the ubiquitin protein ligase MDM2, to deacetylate p53 and allow its degradation (Ito *et al.*, 2002).

The recruitment of proteins that are associated with histone deacetylases, and more importantly those with associations to both HATs and HDACs, to HSV-1 compartments may implicate a more generalized, non-targeted recruitment strategy. Recruitment may be based largely on the acetylation state of the chromatin due to the intranuclear rearrangement of these enzymes in response to HSV-1 infection, rather than the specific recruitment of each individual associated-factor on the basis of their function in relationship to the host and viral replication pathways. Again, the recruitment of these factors may be the result of active recruitment of HATs and HDACs. Altering steady-state activity through infection appears to favor host chromatin silencing. HSV-1 alters the normal distribution of HATs and HDACs, often within permissive chromatin domains. As HSV-1 recruits these proteins it brings with it their affinities for transcriptional coactivator and corepressor complexes, and concomitantly, concentrating binding sites for associated proteins within viral compartments. This possible phenomenon may also be due to global spatial rearrangement of the nuclear matrix, which is known to be associated with many of these factors, and could result in recruitment regardless of functional differences. This will be explored in later experiments (Chapter 3.10).

Table 3.4 The recruitment of HDACs and their potentially associated proteins to HSV-1 compartments.

Protein Recruited to HSV-1 Compartments	Name	Endogenous	Transiently Transfected[†]	Stably Transfected[†]
Histone Deacetylases	HDAC1	+	$+^{f,g}$	N/A
	HDAC2	+	-	N/A
	HDAC3	+	$+^{f,g}$	N/A
	HDAC4	+	$+^{f}$	N/A
	HDAC4 nuclear targeting domain	N/A	$+^{f}$	N/A
	HDAC5	+	-	N/A
	HDAC7	+	-	N/A
Histone Deacetylase Potentially-Associated Proteins	ATM	+	-	-
	Estrogen Receptor	-	$+^{g}$	$+^{g}$
	mSIN3A	+	-	-
	p53	+	-	-
	Retinoblastoma	+	-	-
	Topoisomerase II	+	-	-

+ indicates that an assay for the particular protein was performed
- indicates that an assay was not performed for this protein
N/A indicates the entity is not known to be viable or available
[†] g indicates GFP-protein constructs were used; f indicates FLAG tag-protein constructs were used

3.7 The Activity Histone Deacetylases in Infected Cells

Once the recruitment of HDACs to viral compartments was confirmed, the question turned to the relative activity of HDACs through infection to explain the resultant loss of highly acetylated histones through infection. *In situ* HDAC assays were performed on the same acetylated histone epitopes used for the *in situ* HAT assay, penta-acetylated H4, lysine 9 and 14 of H3, tetra-acetylated H2B and lysine 8 of H4, for comparison. In this assay, cells are cultured for twelve hours and infected in the presence of the reversible HDAC inhibitor Trichostatin A, whose treatment is sustained until one hour prior to harvesting. The removal of the HDAC inhibitor results in a net deacetylation. The remaining acetylation at each epitope was determined by quantifying individual cells by immunofluorescent costaining with an antibody against an acetyl lysine epitope and the immediate HSV-1 immediate early protein ICP4 to evaluate the level of infection as with the *in situ* acetylation and HAT activity assay, described in the Materials and Methods. If HDAC activities are inactivated or decreased, this will result in an increase of total acetylation in these cells due to the failure of HDACs to remove the hyperacetylation induced by the TSA treatment.

During the course of the experimental design phase it was observed that IM cells cultured with TSA had reduced rate of progression through the infection cycle. To achieve comparable infection levels for cells in this assay, cells were infected and cultured for 3, 6 and 9 hours prior to fixation, rather than 2, 4 and 6 hours as with the acetylation and HAT assay. This does not affect comparison of results as the criteria for assessing infection levels, based upon ICP4 staining patterns, remains consistent. Figure 3.16 shows this phenomenon of fewer infected cells and a retarded infection process in IM cells cultured and infected with TSA at 1μg/ml (top panels) versus control cells infected without TSA treatment (bottom) for four hours. Costaining with anti-ICP4 (green) and anti-acetylated H3 (red) reveals that fewer cells were infected and no cells progressed to the highly infected stages when treated with TSA (B) where large areas of the nucleus are occupied with established viral replication compartments, as in the

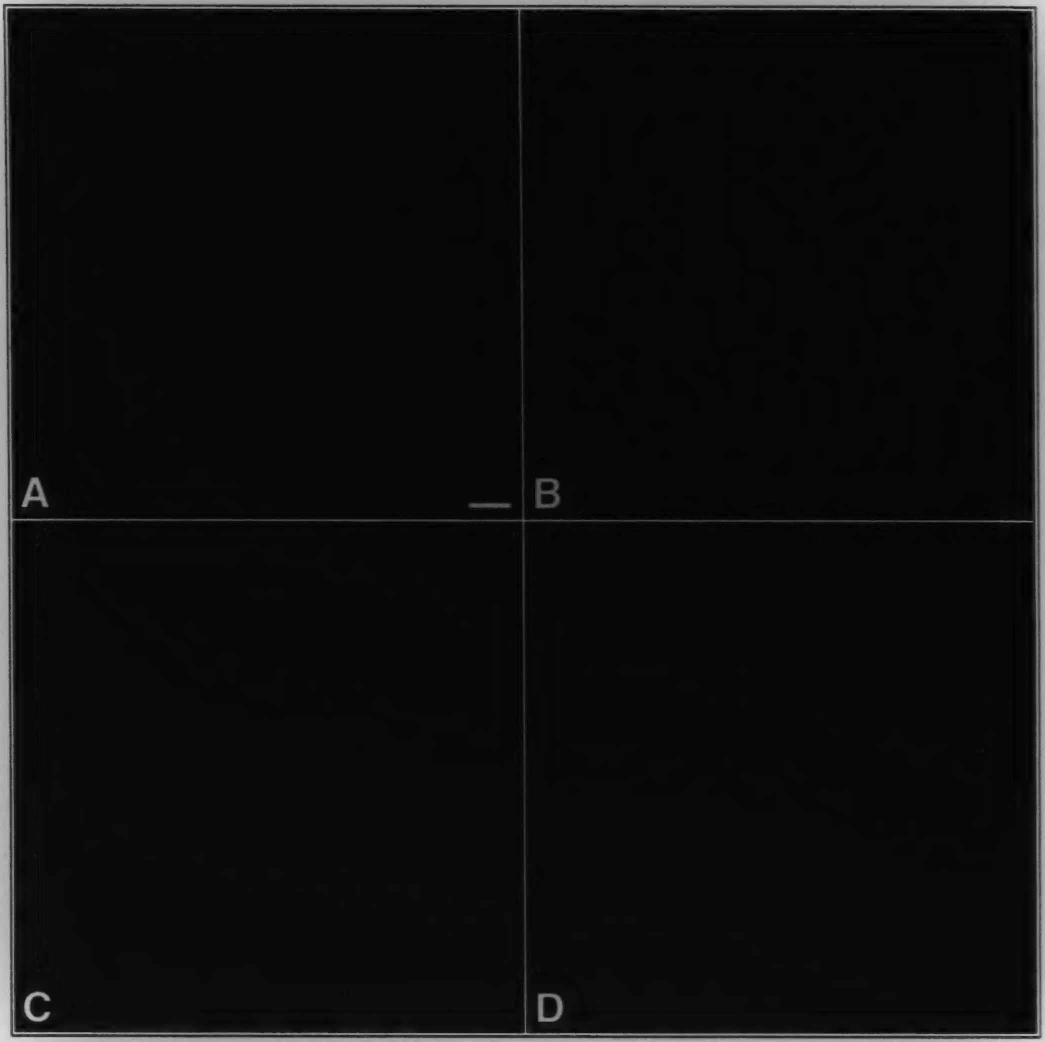

Figure 3.16 Sustained Trichostatin A treatment retards HSV-1 infection. Digital optical sections of Indian Muntjack fibroblast cells infected with HSV-1 and cultured for four hours post-infection were taken. The top panels are a representative example of an *in situ* HDAC assay result. Cells were cultured in 1μg/ml of TSA twelve hours prior to infection, during infection and incubation with the virus and removed 1 hr prior to fixation. The bottom panel is the control, a representative example of an *in situ* acetylation assay result where cells were cultured and infected in the absence of TSA. Cells were stained with the chromatin stain DAPI (blue) and immunofluorescently costained with an antibody against acetylated K9 and K14 of H3 (red) and the HSV-1 immediate early protein ICP4. Field-of-view images of cells with ICP4 staining alone show a range of low infected cells to highly infected cells with large, established replication compartments in the control cells (D). In contrast, cells cultured in TSA show fewer infected cells and productive infections that have only advanced past the prereplicative foci stage (B). The scale bar represents 10 μm.

control cells (D). As expected, in control cells highly acetylated H3 species are lost (C) as compared to those treated with TSA (A) in directly proportional relationship to the level of infection. This inhibition of immediate early protein expression with treatment of TSA contradicts corroborates several earlier studies into histone deacetylase inhibitors and viral gene expression (Preston and McFarlane, 1998; Hobbs and DeLuca, 1999).

Some subtle changes in the relative HAT and HDAC activities were observed in the results of the *in situ* HDAC activity assay (Table 3.5). The low infection stages were normalized to 1.0 as with the HAT assay for comparison. The penta-acetylated hv1 antibody, which recognizes highly acetylated species of histone H4, shows evidence of reduced HAT activity in cells with high infection levels (p=0.0011) but not HDAC activity. Interestingly, there is also an apparent net increase in HDAC activity specific for acetylated histone H3 to coincide with elevated HAT activity on H3 in the medium stages of infection (Table 3.3). This observation does not completely corroborate with the *in situ* acetylation data where HDAC activity seems to be slightly favored over HAT

Table 3.5 *In situ* assay for HDAC activity detected for specific acetyl lysine epitopes in Indian Muntjack fibroblast cells.

Acetylated Lysine Residues	Infection Level	n	Mean Acetylation/ DAPI +/-SD	p-value	Statistical Significance
Penta-acetylated H4	Low	30	1.000 +/- 0.498	n/a	
	Medium	80	0.689 +/- 0.334	p=0.0003	+++
	High	62	0.556 +/- 0.278	p<0.0001	+++
K9 and K14 of H3	Low	107	1.000 +/- 0.415	n/a	
	Medium	63	0.774 +/- 0.412	p=0.0007	+++
	High	57	0.571 +/- 0.247	p<0.0001	+++
Total H2B	Low	159	1.000 +/- 0.443	n/a	
	Medium	80	0.773 +/- 0.392	p=0.0001	+++
	High	68	0.610 +/- 0.347	p<0.0001	+++
K8 of H4	Low	89	1.000 +/- 0.635	n/a	
	Medium	80	1.153 +/- 0.604	p=0.1114	-
	High	39	1.019 +/- 0.775	p=0.8846	-

activity (Table 3.2) but enzyme activity and acetylation data is reconciled for highly infected cells. Histone H2B HAT activity, but not HDAC activity, also appears to be reduced during infection. These results indicate that although there are subtle differences between specific epitopes that might be a reflection of small differences in behavior and recruitment of specific HATs and HDACs during infection, in general, the *in situ* HAT and HDAC activities are biased towards deacetylation of the highly acetylated transcription associated epitopes (penta, AcH3, and AcH2B) but not the bulk acetylation levels, represented by acetylation of histone H4 at lysine 8, during the course of infection. This is consistent with the participation of HATs and HDACs in the repression of the host genome post infection.

To further support the *in situ* HDAC assay data, an *in vitro* HDAC assay was performed using whole HeLa S3 cell enzyme extract to deacetylate hyperacetylated [^{3}H] acetyl lysine-histone substrate, the results of which are shown in Figure 3.17. There was no significant difference in HDAC activity between non-infected cells and cells at 2, 4, 6, and 9 hours of infection, from small prereplication compartments to the complete recruitment of HDACs to viral compartments. Vertical bars represent standard error of the mean. This data shows total HDACs maintain activity through infection. At 12 hours post-infection, there is an expected decrease in activity that coincides with late gene expression and the timing of the shutdown of RNAPII for host gene expression (Spencer *et al.*, 1997), verifiable with fluorouridine labeling of RNA transcripts (data not shown). Treatment with 400 nM TSA completely inhibited HDAC activity to background levels and demonstrated that activity released was not due to proteolysis of the substrate.

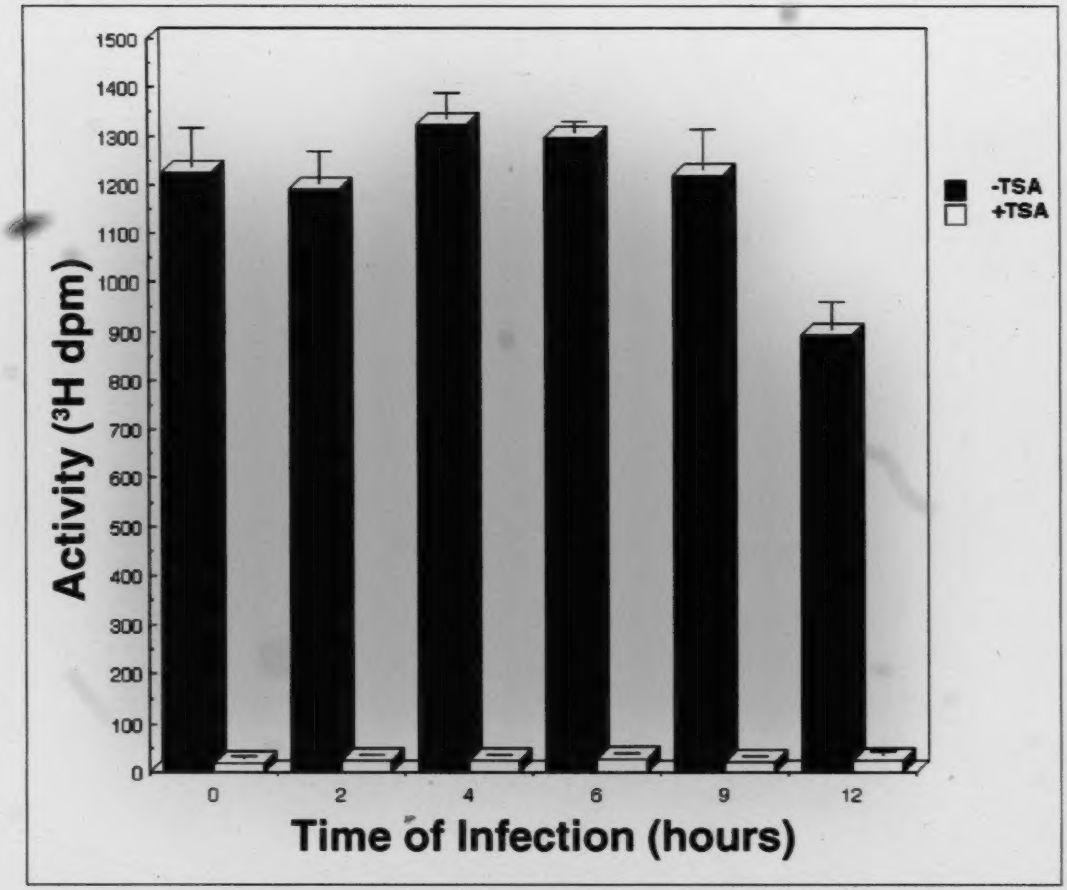

Figure 3.17 Histone deacetylase activity is maintained through the recruitment stages to viral replication compartments. Histone deacetylase activity *in vitro* is measured through 0, 2, 4, 6, 9 and 12 hours of infection with HSV-1. Cellular extracts of HeLa S3 cells were tested for HDAC activity by measuring (in dpm) the release of [^{3}H]acetate from a hyperacetylated [^{3}H]acetyl lysine-histone preparation in the presence or absence of 400nM TSA. HDAC activity is significant and consistent between non-infected cells and cells infected through 9 hours. The decrease in activity 12 hours post-infection is attributed to HDACs' spatial-temporal correlation with transcriptional shutdown of host genes. Vertical bars represent standard error of the mean.

3.8 HSV-1 Preferentially Recruits Certain Proteins

Preferential recruitment of HATs over HDACs may account for the decrease in class I transcription related acetylation through infection and explain the consistent, unchanged relative HDAC activity and comparatively consistent but slightly varied relative HAT activity on particular hyperacetylated histone epitopes. CBP/p300 and class I HDACs have been observed colocalizing with HSV-1 very early in infection, relative to most other non-HAT or HDAC entities that mainly tend to recruit into well-established replication compartments. Figure 3.18 shows SK-N-SH neuroblastoma cells infected for three hours. As viral compartments are not distinctly discernable as clearings in the

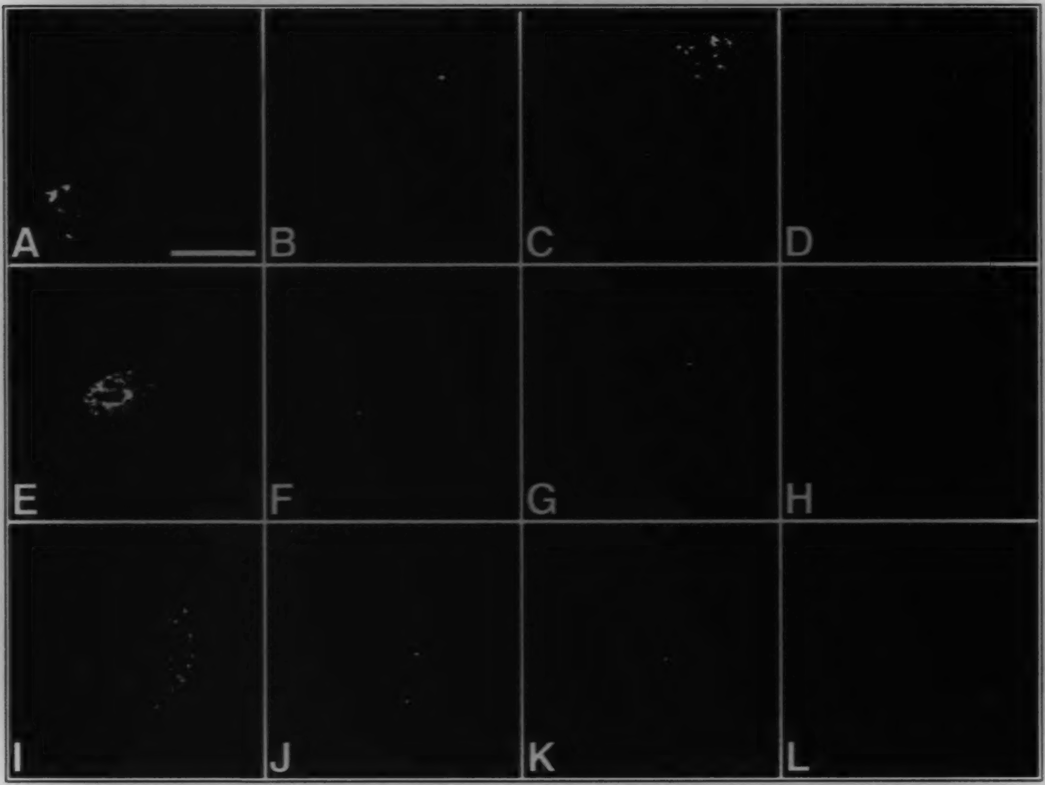

Figure 3.18 The differential recruitment of HATs and HDACs to prereplicative foci *in situ*. The histone deacetylase CBP is recruited to viral prereplication compartments in early stages of HSV-1 infection while HDAC1 and HDAC4, class I and II deacetylases respectively, are not. Digital optical sections of SK-N-SH neuroblastoma cells infected with HSV-1 and incubated for three hours prior to fixation were taken. Infection levels cannot be ascertained at early infection stages with the chromatin stain DAPI (A, E, I). Cells were costained with antibodies against ICP4 to detect viral infection (B, F, J) and against the C-terminus of CBP (C), anti-HDAC1 (G) and anti-HDAC4 (K). The composite image of ICP4 and CBP (D) shows an enrichment of CBP (red) in a small focus colocalizing with an ICP4 (green) prereplicative focus, but no discernable alteration of the overall CBP staining pattern in the remainder of the nucleus when compared to the uninfected cell in the field-of-view. The composite image of ICP4 and HDAC1 (H) reveals abnormal HDAC (red) enrichment in foci that do not colocalize with ICP4 (green) prereplicative foci. The composite image of HDAC4 (red) and ICP4 (green) does not show any apparent correlation between the two factors. The scale bar represents 10 μm.

chromatin when stained with DAPI (A, E, I), anti-ICP4 staining (B, F, J) reveals the low infection level, characterized by a diffuse anti-ICP4 staining pattern and several small prereplicative foci. Immunofluorescent punctate staining patterns remain relatively typical for the C-terminus of CBP (C), the class I histone deacetylase HDACI (G) and class II histone deacetylase HDAC4 (K) when compared to mock infected cells. Composite images show that CBP (red) and ICP4 (green) prereplicative foci colocalize and overlap at early stages of infection (D) whereas small HDAC1 foci (red) do not colocalize with ICP4 (green) prereplicative foci (H) and there is seemingly no correlation between HDAC4 (red) and ICP4 (green) in the composite image (L). This may indicate that HATs are recruited to areas where parental viral genomes exist over HDACs. This differential recruitment of HDACs may be based on their class and functionality within or apart from corepressor complexes, which are seen to recruit at later stages of HSV-1 infection. This result could be expected as there is to date a lack of evidence associating HSV-1 proteins with HDACs or their complexes. Anti-CBP at the C-terminus does not label CBP in PML bodies, which are known to colocalize, but not overlap with, HSV-1 prereplicative compartments (Lopez *et al.*, 2002).

This preferential recruitment of HATs over HDACs to sites of viral replication does not appear to be a generalized phenomenon for all HAT families. The MYST family of HATs is recruited into viral replication compartments once productive compartments are established. Figure 3.19 of an SK-N-SH cell with a low infection level, having several prereplicative compartments labeled with anti-ICP4 (B), is sufficient to alter the normal punctate nuclear staining in interphase cells observed for the MYST acetyltransferase family (C). Interestingly, in the composite image (D), ICP4 (green) and some highly enriched MYST (red) foci colocalize but do not overlap as with CBP. These large MYST foci, either associated or unassociated with ICP4 foci, occupy atypically enlarged interchromatin areas that are devoid of the chromatin stain DAPI (blue). This suggests an alteration of HAT distribution in the nuclear space and chromatin reorganization exists very early in the viral infection process. Initial enlargement of the intranuclear space may be attributed to the recruitment or redistribution of endogenous intranuclear factors and not completely a result of viral gene products occupying the interchromatin space.

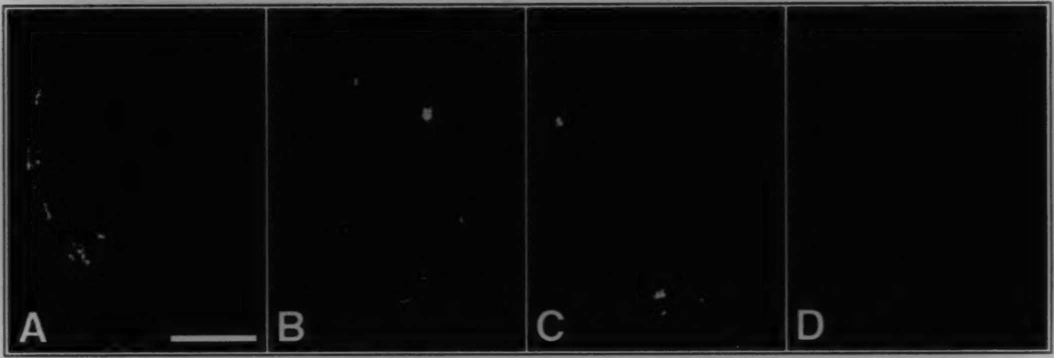

Figure 3.19 MYST family proteins are recruited by but do not overlap with HSV-1 prereplicative compartments at low infection levels. Digital optical sections were taken of SK-N-SH neuroblastoma cells infected with HSV-1 and incubated for three hours prior to fixation. The cells were stained with the chromatin stain DAPI (A) and immunofluorescently costained anti-ICP4 and anti-MYST MOZ motif. The composite image (D) reveals colocalization of some large abnormal MYST (red) foci with ICP4 (green) prereplicative foci. Both MYST foci alone and associated with ICP4 foci occupy large areas of interchromatin space devoid of the chromatin stain DAPI (blue). The scale bar represents 5 µm.

Alterations in the distribution patterns of CBP/p300 and class I HDACs appeared earlier than class II HDACs and also preferentially over other acetyltransferases. An early change in steady state equilibrium of these enzymes could cause changes in histone acetylation. An *in vivo* assay was devised for the purpose of quantitating confocal data to determine the relative recruitment of CBP, p300 and HDAC1 to small prereplicative sites through to large annexed compartments. *In situ* recruitment assays (Figure 3.20) showed the significant, preferential enrichment of HATs over HDACs in viral compartments

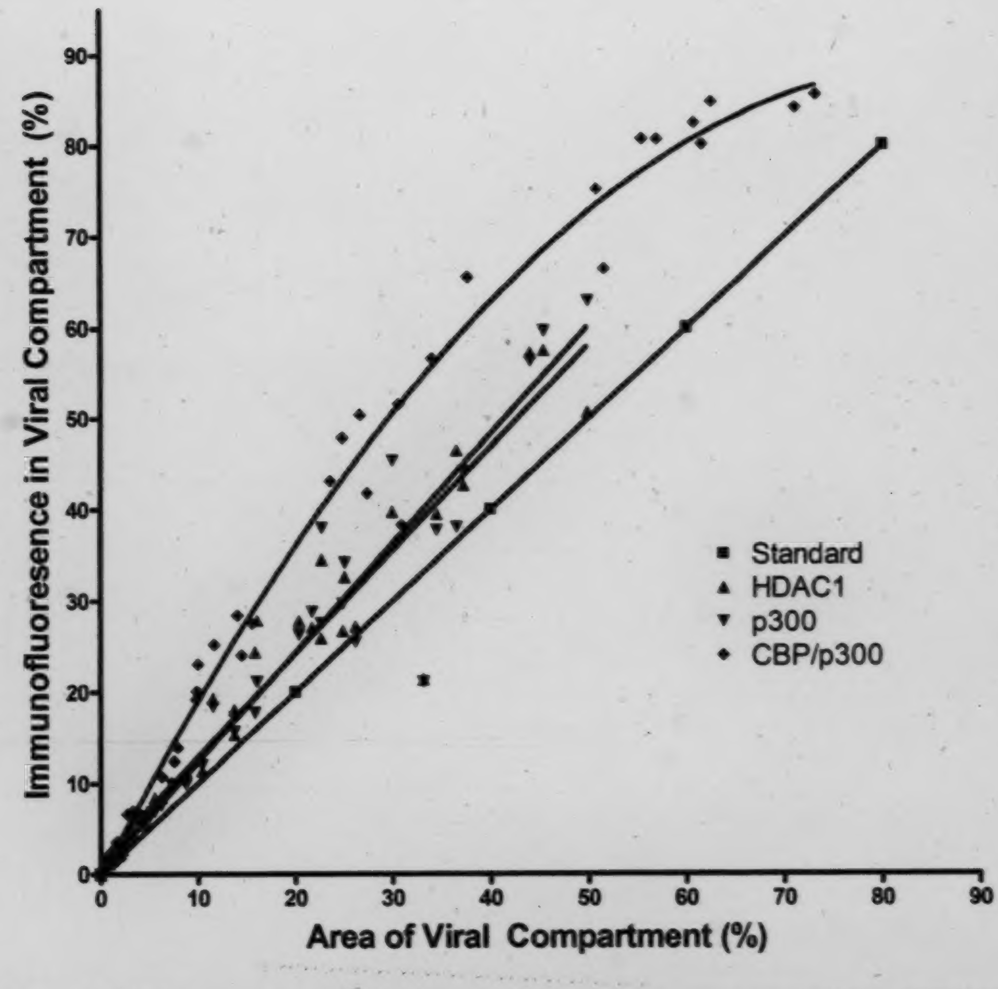

Figure 3.20 CBP is preferentially recruited to HSV-1 compartments over p300 and HDAC1. *In situ* data was collected from immunofluorescently labeled SK-N-SH cells and data analyzed from confocal images as described in Materials and Methods. A linear relationship results when the percentage of immunofluorescence contained within viral compartments is plotted versus the percentage area of the nucleus occupied by the compartment for the proteins HDAC1 and p300. A non-linear relationship exists for the recruitment of CBP/p300. A standard line with a slope equal to 1 represents non-preferential recruitment. Resultant curves for p300 and CBP/p300 have significantly greater slopes than the standard. The resultant slope for HDAC1 is not statistically greater than the standard. The CBP/p300 antibody, recognizing both p300 and its homolog CBP, has significantly greater recruitment than p300. Therefore, CBP is preferentially recruited to viral compartments over p300. Complete statistical analysis is found in Table 3.5.

compared with the rest of the nucleus in SK-N-SH cells. The percentage of fluorescence for HDAC1, p300 or CBP/p300 in viral compartments was plotted versus the percentage of area occupied by the viral compartment in the nucleus as described in Materials and Methods. The CBP/p300 antibody, recognizing both p300 and its homolog CBP, has significantly greater recruitment than p300 alone. CBP/p300, due to their considerable functional redundancy and homology, are hypothesized to coincidently enrich in normal cells in order to facilitate concurrent signaling cascades. Only recently evidence for some separate functional identities of these proteins had been uncovered. The preferential recruitment of CBP is shown by the significant increase in slope of CBP/p300 over p300 alone. Therefore, the difference in the graphical relationship is due to CBP and will henceforth be referred to as CBP for this assay.

Statistical analyses of the recruitment data plot were performed to determine the significance of the results in terms of fitting the regression model and identifying preferential recruitment (Table 3.1). Linear regression analysis was performed and it was determined that the recruitment of HDAC1 and p300 were not significantly nonlinear as determined by the high P values obtained from the runs test, which tests for significant nonlinearity (P_{HDAC1}=0.7857; P_{p300}=0.4517). Both proteins significantly fit the linear model (squares of the correlation coefficient [r^2] were 0.9331 and 0.9363 respectively). The graph for CBP was significantly non-linear ($P_{CBP}< 0.0001$) and a nonlinear regression was performed using a second order polynomial equation. The deviation from the model was determined not to be significant by the runs test ($P_{CBP}= 0.5614$). The sharp rise of the curve until approximately 50% of the nucleus is filled by viral compartment may signify that CBP is actively recruited to viral compartments very early and in a different manner than p300. As the infection progresses, the relatively active recruitment of CBP is decreased.

Table 3.6 Statistical summary of the preferential recruitment assay graph in Figure 3.20.

Analysis	CBP/p300	p300	HDAC1
Regression Model	non-linear	linear	linear
Slope	n/a	1.093 to 1.308	1.029 to 1.237
Goodness of Fit			
Degrees of Freedom	44	n/a	n/a
R^2	0.9898	0.9363	0.9331
$s_{y.x}$	3.163	4.594	4.450
Absolute Sum of Squares	440.1	n/a	n/a
Runs Test			
P value	0.5614	0.4517	0.7857
Deviation from Model	not significant	not significant	not significant
Comparison to Standard			
F_{slope}	standard does not fall within 95% confidence interval of the curve	5.38517	2.52913
P_{slope}		0.02577	0.12
Difference between slopes		significant	not significant
$F_{elevations}$		n/a	6.4425
$P_{elevations}$		n/a	0.01525
Difference between elevations		n/a	significant

When compared to a standard with a slope of 1, representing neither recruitment nor exclusion from viral compartments, the HATs show significant recruitment to viral compartments while HDAC1 does not (Table 3.6). Additionally, HDAC1 is the major nuclear HDAC. It is the most abundant and thought to be the most multifunctional, implicated in many nuclear processes as a member of many functionally distinct multiprotein complexes (Gray and Ekström, 2001). As such, loss of steady-state acetylation through infection may be explained through the preferential recruitment of CBP and p300 relative to HDAC1.

3.9 The nature of CBP in viral replication compartments

Recently, the intranuclear compartmentalization of HSV-1 has been studied in real time from both the perspective of the host chromatin and the viral compartment itself using GFP-fusion proteins of histone H2B and the immediate early protein ICP8 respectively (Monier *et al.*, 2000; Taylor *et al.*, 2003). Preliminary results of Fluorescence Loss in Photobleaching (FLIP) and Fluorescence Recovery After Photobleaching (FRAP) experiments using the protein CBP show the dynamic nature of proteins within the viral compartment.

In Figure 3.21, FLIP demonstrates connectivity between CBP in the viral compartment and the nucleoplasmic population. Enrichment of transiently expressed CBP-GFP in well-established viral compartments (v.c.) was easily identifiable in the FITC channel (488 nm excitation wavelength). This was confirmed with Hoechst staining visualized with low-intensity IR multiphoton excitation revealed chromatin clearing in the same area (data not shown). A 2 μm diameter region was selected in the approximate centre of the cell (to negate boundary effects of the membrane particle diffusion) in a region of the nucleoplasm that was not occupied by viral compartments but equidistant from viral compartment borders. The cell was bleached repeatedly with five consecutive high-intensity laser pulses (100%), allowed to recover for five seconds as determined empirically from preliminary trials, so as to allow for the equilibration of the CBP-GFP particles, and imaged using low-intensity (1%) laser power after this first bleach recovery cycle (b1). The bleach pulse and recovery cycle was immediately repeated and recorded after each cycle (b2, b3, b4 and b5). The decrease in CBP-GFP signal intensity of the viral compartments through the bleach-pulse cycles shows that

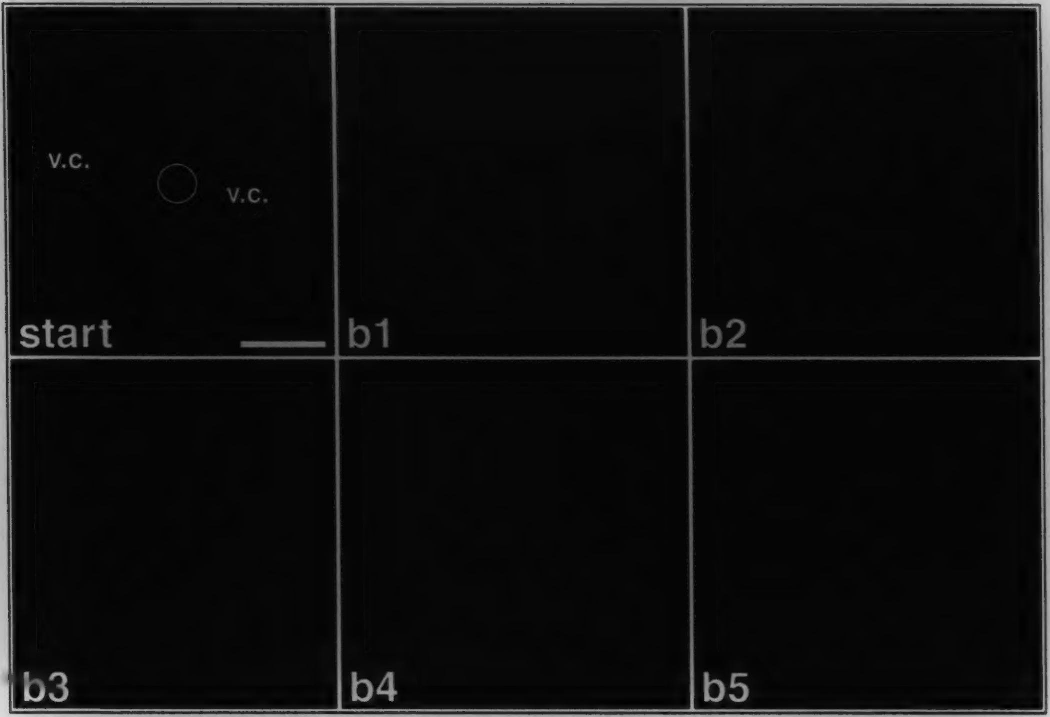

Figure 3.21 FLIP in a CBP-GFP transiently transfected cell and infected with HSV-1. HISM cells were transfected and incubated twelve hours prior to infection. Cells were infected with HSV-1 and incubated for six hours prior to mounting. A 2 μm diameter circle (indicated in the start panel) in the nucleoplasm outside of viral compartments (v.c.) was bleached five times successively and allowed to recover for five seconds. Cells were imaged after each bleach pulse and recovery cycle, beginning with the first (b1, b2, b3, b4, and b5). The scale bar represents 5 um.

CBP is not stably bound within viral compartments. It is dynamic, in constant flux and exchanging with the nucleoplasmic population. At steady-state, CBP is preferentially enriched within viral compartments. As the overall CBP-GFP signal decreases, enrichment within viral compartments is still visible. This result was also observed in cells stably transfected with CBP-GFP.

Figure 3.22 shows the results of a FRAP experiment using IM cells that are transfected with CBP-GFP and then infected with HSV-1 to low infection levels. An area 2 μm in diameter surrounding a prereplicative focus and another in the nucleoplasm of the same size and the same Y and Z coordinates were selected for bleaching. The circles were equidistant from the nuclear membrane to negate any differential membrane boundary effects on particle diffusion. The areas were bleached using 100% laser intensity and imaged using low-intensity (1%) laser scans at intervals determined empirically through preliminary photobleaching trials until CBP-GFP dynamics recovered and stabilized. The prereplicative focus is completely bleached and recovers slower (at approximately 90 seconds) than that of the nucleoplasmic bleached area (at approximately 30 seconds; not corrected for photobleaching). While further quantitative analysis is required to confirm and completely characterize these findings, it does suggest that CBP populations within the viral compartment are less mobile, or have a smaller effective diffusion coefficient, compared to nucleoplasmic populations. Similar preliminary experiments were performed using stable and transiently transfected cells

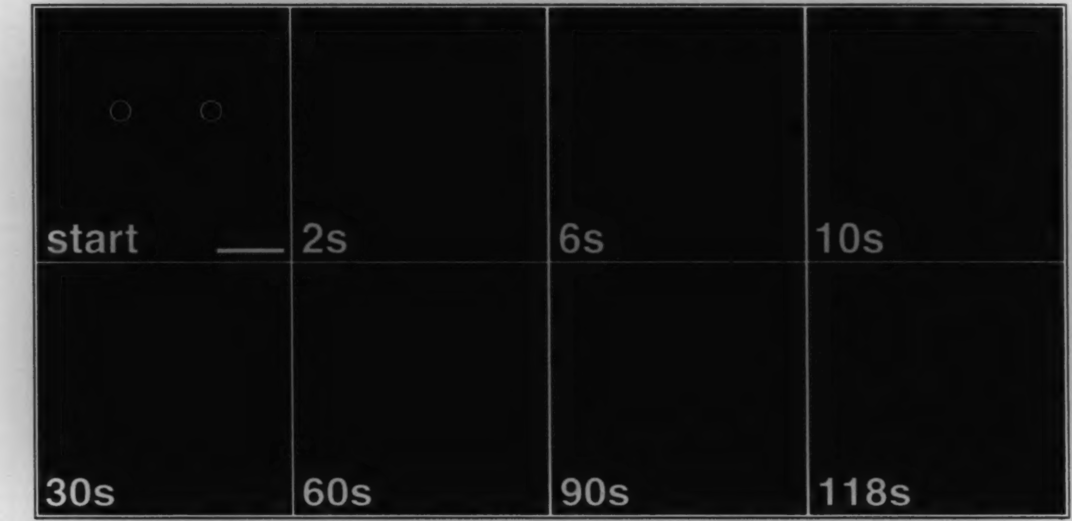

Figure 3.22 FRAP in a CBP-GFP transiently transfected cell and infected with HSV-1. Indian Muntjack fibroblast cells were transfected and incubated twelve hours prior to infection. Cells were infected with HSV-1 and incubated for three hours prior to mounting. A 2 µm diameter circle encompassing a viral prereplicative foci and an identical circle in the nucleoplasm at the same Y and Z coordinates (circles in start panel) were bleached by a single laser pulse. The cell was imaged at the indicated times after bleaching until recovery of the bleached area was observed. The scale bar represents 5 µm.

expressing AP-2-GFP showing similar characteristics of dynamic nuclear compartmentalization as with CBP-GFP (data not shown), although no direct comparisons were made.

3.10 Nuclear structural proteins, with undetermined association to HSV-1, are recruited to viral compartments

To determine whether or not compartmentalization to viral compartments is a global phenomenon, I tested additional antibodies recognizing a component of nuclear speckles (splicing factor compartments [SFCs] or interchromatin granule clusters [IGCs]) SC-35 and the nuclear mitotic apparatus (NuMa). While these proteins are not known to be directly associated with HATs or HDACs, they are believed to be associated with the nuclear matrix. Both were recruited to viral compartments.

Along with chromatin and nucleoli, the most prominent intranuclear structures are the SFCs. They accumulate host and viral RNAs and RNAPII transcription is thought to take place upon their periphery. These are the source of splicing factors for surrounding gene loci and their periphery is also enriched in HAT and HDAC activity (Hendzel *et al.*, 1998). As demonstrated in SK-N-SH cells in Figure 3.23, upon HSV-1 infection, these structures are recruited to viral compartments in two distinct patterns. One anti-SC-35 (green) staining pattern is diffuse (A) while the other resembles the native punctate patterns of a smaller size inter dispersed with diffuse areas (B). The normal SC-35 punctate distribution pattern with large foci has been characterized (Hendzel *et al.*, 1998) and is represented in this experiment in panel C. The cells were immunofluorescently costained with anti-acetylated K12 of H4 (red), a species present in moderately acetylated

Figure 3.23 HSV-1 recruits splicing factor compartments and creates distinct altered staining patterns. Digital optical sections were taken of SK-N-SH neuroblastoma cells infected with HSV-1 and incubated for six hours prior to fixation. Cells were stained with the chromatin stain DAPI (blue) to show viral compartments. Immunofluorescent staining with acetylated lysine 12 of H4 confirmed areas occupied by viral compartments. Anti-SC-35 antibodies (green) revealed a diffuse staining pattern (A) and a punctate staining pattern (B) for splicing factor compartments. Composite images show speckles in areas devoid of chromatin. Typical SC-35 staining patterns for uninfected cells are shown in panel C. The scale bar represents 10 μm.

chromatin, to confirm the presence and position of viral compartments in conjunction with the DAPI staining (blue). Composite images show recruitment of splicing factors, which compose splicing factor compartments, to the viral compartment, devoid of chromatin and acetylated histone species. A similar result of altered *in situ* SC-35 distribution was reported for herpesvirus saimiri by an interaction with a protein homologous to ICP27 of HSV-1, implicated in inhibiting host splicing (Cooper *et al.*, 1999).

Upon further inspection it appears that nuclear speckles, despite the altered distribution and staining pattern with HSV-1 infection, retain a spatial relationship with the HAT CBP (Figure 3.24). The pattern of recruitment of CBP (C) to the viral compartment is consistent with that presented in Figure 3.2 and the SC-35 staining pattern (B) resembles that of Figure 3.24B. As aforementioned, speckles maintain similar distribution patterns with areas of dynamic acetyltransferase activity, although none is detected in the viral compartment. The composite image confirms the existence of both proteins within the viral compartment, devoid of DAPI (blue), but reveals that CBP foci (red) encroach upon, but do not overlap with, areas occupied by SC-35. This may be indicative of organization of factors in the viral compartment or the maintenance of distinct territories based upon exclusionary properties of the two proteins. Speckles are believed to be associated with the nuclear matrix. However, various evidence suggests that some level of organized RNAPII transcription and splicing events may exist in the absence of nuclear matrix (Hendzel *et al.*, 1999; Wei *et al.*, 1999) and therefore the presence of a traditional nuclear matrix per se may not be necessary for transcription events to occur within the viral compartment.

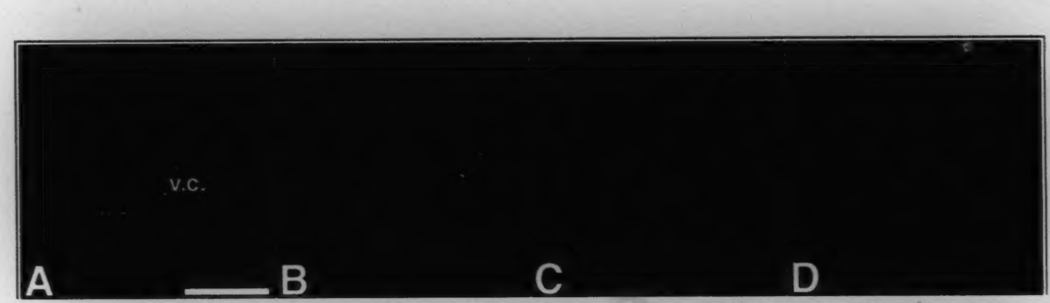

Figure 3.24 Splicing factor compartments and CBP maintain a spatial relationship within HSV-1 compartments. Digital optical sections were taken of Indian Muntjack cells infected with HSV-1 and incubated for six hours prior to fixation. Cells were stained with the chromatin stain DAPI (A) to reveal areas occupied by the viral compartment (v.c.). Cells were immunofluorescently costained with antibodies against SC-35 (B) and the C-terminus of CBP (C). The composite image shows SC-35 (green) and CBP (red) occupy distinct areas of the intercompartment space, devoid of chromatin (blue). The scale bar represents 10 μm.

The Nuclear Mitotic Apparatus (NuMA), in mitosis, is a centrosomal component required for the organization and stabilization of spindle poles. However, there is evidence that NuMA in the interphase nucleus functions as a scaffold protein (Harborth and Osborn, 1999). NuMA, in the normal interphase nucleus, displays a diffuse distribution (Taimen and Kallajoki, 2003). HSV-1 recruits NuMA, another protein with an undefined relationship with either HATs or HDACs, to viral compartments in the late stages of infection (data not shown). Rearrangement of these putative structural proteins during HSV-1 infection, in combination with the recruitment of HATs, HDACs and other proteins known to be associated with a nuclear karyoskeleton, may more truly parallel host transcription associated nuclear organization and describe a means by which viral infection annexes nuclear space and furthers the shift toward viral processes over host function. Recent studies suggest, however, that certain viruses, during virus-induced cell death, cleave NuMA and the proteolytic product remains around nuclear fragments (Taimen *et al.*, 2004). Therefore, whether NuMa is recruited to viral compartments as an intact protein or a cleavage product is not known. This considered together, the deconstruction of PML and speckles through infection may make the state of recruited NuMA of less consequence. Regardless of the state in which NuMA is recruited, there is a global redistribution of nuclear matrix-associated proteins, the normal organization of the nucleus is disrupted and host transcription is decreased as a result of HSV-1 infection.

3.11 Proteins that are not recruited to viral compartments

The control results for recruitment of eGFP and all acetyl lysine and histone residues were negative. In addition, only one other protein tested for recruitment, Protein Kinase $A_{RIA\alpha}$, was not recruited to HSV-1 viral compartments. This protein is known to phosphorylate CREB and its subcellular localization is dependent upon its activation status in response to the cAMP cell signaling pathway (Shaywitz and Greenberg, 1999). Therefore, this protein may be categorized as having an indirect relationship with histone acetyltransferases through CREB activation. In Figure 3.25, $PKA_{RIA\alpha}$ (C, red) does not exhibit the same immunofluorescent staining pattern in infected cells as in uninfected

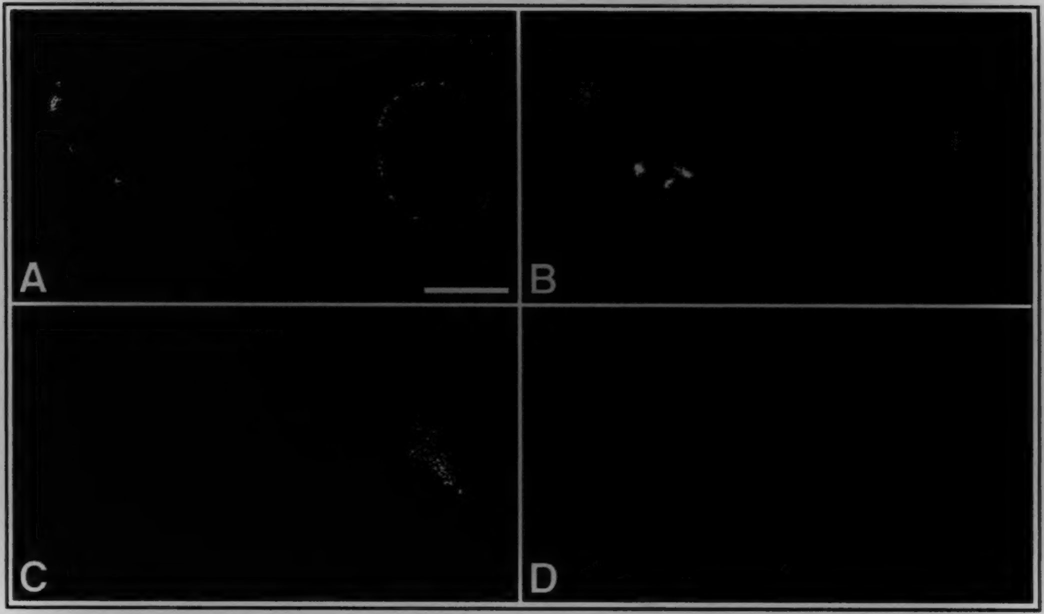

Figure 3.25 The nuclear distribution of PKA$_{RIA\alpha}$ in an uninfected and low-infected cell. Digital optical sections were taken of SK-N-SH neuroblastoma cells infected with HSV-1 and incubated for three hours prior to fixation. Cells were stained with DAPI (A) and immunofluorescently costained with anti-ICP4 (B) and anti-PKA$_{RIA\alpha}$ (C). The composite image (D) shows the relationship of the chromatin (blue) to the diffuse ICP4 staining (green) in the infected cell and the distribution of PKA$_{RIA\alpha}$ (red) in both cells. PKA$_{RIA\alpha}$ shows altered distribution, primarily nucleolar, in the infected cell. The scale bar represents 10 μm.

cells. This factor does not colocalize in the same interchromatin region with the ICP4 (green) diffuse staining pattern, indicative of a low infection level, as is typical of all other proteins tested that are positive for recruitment. It also appears that there is reduced PKA$_{RIA\alpha}$ signal in the nucleus. The entire immunofluorescent signal present in the nucleus is present in the nucleolus, identified as a region of dense heterochromatic staining in the DAPI channel (A) and areas where ICP4 is excluded (B). This staining pattern persists and the immunofluorescent signal decreases in intensity until the nucleolus is disrupted at high infection levels (data not shown). This may be expected as PKA is known to be associated with the nucleolus, interchromatin granules, and coiled bodies *in situ*. PKA is also known to shuttle between the cytoplasm and nucleus in response to certain stimuli and nuclear concentrations are related inversely with the amount of condensed chromatin (Trinczek *et al.*, 1993).

4. DISCUSSION

4.1 The decrease of transcription-associated histone acetylation is a result of viral recruitment of histone acetyltransferases and deacetylases

The activity of histone acetyltransferases and deacetylases is essential for the regulation of host gene transcription. A shift in the dynamic equilibrium of HAT and HDAC activities results in the net acetylation or deacetylation of chromatin respectively. The steady-state distributions of these enzymes are spatially regulated, and may be mediated by their association with nuclear karyoskeletal elements (Hendzel *et al.*, 1998). Theories regarding spatial regulation have evolved to accommodate the realization that functions within the nucleus are compartmentalized. Nuclear compartments within the interchromatin space are enriched in specific resident proteins and maintain a characteristic morphology and number. They are distinct from cytoplasmic compartments in that these components are not membrane-bound. The precise mechanism behind compartment formation has yet to be completely elucidated and remains controversial (Hendzel *et al.*, 2001). It is known, however, that nuclear compartments are dynamic in nature, altering shape and composition as a result of gene function (Misteli, 2001).

HSV-1 infection is an excellent model because it allows one to modify nuclear organization by forcing the establishment of “artificial” nuclear compartments in order to study the alteration of processes from the normal state (Hendzel *et al.*, 2001). HSV-1 utilizes the host transcriptional machinery to transcribe the viral genome. As RNAPII is increasingly recruited to viral replication compartments during the lytic cycle, host transcription is repressed and eventually lost (Rixon *et al.*, 1983; Puvion-Dutilleul *et al.*, 1985; Rice *et al.*, 1995; Jenkins and Spencer, 2001). The promoters of early and late viral genes are indistinguishable from those of the host genome (Cheung *et al.*, 1997), and while there are a low number of viral genomes present early in infection, their genes are preferentially transcribed. The mechanism of RNAPII recruitment to facilitate viral transcription remains unclear. Given that the direct study of the RNAPII holoenzyme is extremely complicated due to its multi-factorial nature, I instead examined the activity of HATs and HDACs in the context of their spatial distribution. Understanding the nature of these integral regulators of transcription sheds light on the process of transcription in terms of nuclear organization.

One may assume that HSV-1 requires factors relevant to RNAPII transcription and cell cycle regulators to be recruited to viral genomes in order to transcribe viral genes. Initially, I observed that representatives from all histone acetyltransferase families assayed were recruited to viral compartments. In addition, all proteins with direct associations to HATs were recruited to viral compartments. Generally, as infection progressed and viral replication compartments grew to sizes correlating with high levels of infection, recruited factors appeared more concentrated in viral compartments compared to the rest of the nucleus. This could suggest either a more active recruitment of factors by HSV-1 gene products as infection progresses or a diffusion-based enrichment of factors within the annexed and expanded interchromatin space.

There is abroad range of HATs recruited to viral compartments and the concomitant recruitment of their associated proteins may be as a result of being substrates for catalysis or constituents of coactivator complexes. One exception that I have discovered is a protein that is associated with HATs that is not recruited. Protein Kinase

$A_{RI\alpha}$ is associated with HATs due to its ability to phosphorylate CREB. Its localization in the nucleus is functionally determined by its activation status in response to cAMP cell signaling, which leads to the phosphorylation of CREB (Shaywitz and Greenberg, 1999). Instead of occupying interchromatin space, $PKA_{RI\alpha}$ is detected in the nucleolus early in infection prior to the disruption of the nucleolus. Transcription factors and transactivators associated with RNAPII mRNA production are mainly recruited. However, $PKA_{RI\alpha}$ is associated with rRNA transcription and, therefore, the lack of PKA recruitment is consistent with the continuance and gradual decline of RNAPI activity through viral infection. Such nuclear distribution shows the preservation of a nuclear compartment functionally insignificant to viral replication early in infection before global host chromatin rearrangement at high infection levels. This suggests that the recruitment of proteins needed by the virus, at least initially, may be specialized or functionally targeted.

Despite the recruitment of proteins that are functionally associated with or substrates of HATs for factor acetyltransferase activity, no acetyl lysine residues were detected within these compartments. This suggests that any recruitment of HATs to viral compartments, actively or passively, is not for the purpose of acetylating constituent proteins. Viral compartments do not contain chromatin and there is no evidence for a chromatin-like structure in viral compartments (Spencer *et al.*, 2000). This was confirmed by the absence of DAPI and Hoechst 33258 DNA staining. Additionally, all immunofluorescence assays failed to detect any acetyl lysine epitopes in the viral compartments. All acetylated histone species were excluded as well. This absence of chromatin structure most likely disrupts any normal HAT functional activity and may interfere with protein-protein associations or disrupt complex nucleation or targeting events. The loss of observable HAT activity could also be due to enzymatic inhibition or inactivation. Alternatively, recruitment of HATs may serve as a means through which the virus sequesters HATs away from endogenous promoters and shuts down host cell transcription.

I confirmed that a decrease in transcription correlated generally with an increase in the volume of viral replication compartments and, therefore, with the recruitment of HATs. The acetylation state of chromatin confirms that dynamic histone acetylation, which is associated with transcription, is decreased by HSV-1 infection. *In situ* HAT activity assay results, primarily for acetylated K9 and K14 epitopes on H3, indicate that HATs remain active through high levels of infection. To coincide with these results, H4 deacetylation has been observed in bovine herpesvirus infection. This deacetylation is mediated by VP22 binding, a viral tegument protein with conserved homology within the alphaherpesvirus family and implicated in nuclear targeting of the virion (Ren *et al.*, 2001)but see (Harms *et al.*, 2000). Also, there is no alteration in acetylation levels for K8 of histone H3 through infection suggesting that some intermediate HAT activity is retained through infection. This level of acetylation may be brought about by a combination of HAT and HDAC activities (class II acetylation) that are not specifically targeted to chromatin but are characteristic of permissive regions of chromatin (Vogelauer *et al.*, 2000). Throughout the infection process, permissive chromatin domains may be somewhat maintained until late in infection, when viral compartments annex the interchromatin space and host chromatin condenses. Therefore, a decrease in RNAPII activity may not be attributed to a loss of global, class II acetylation. The loss of histone acetylations relevant to transcriptional activation (class I acetylation) coincide with host RNAPII shut down. This gives support to the multifaceted model of gene activation where intermediate acetylation states are a prerequisite for the creation of permissive chromatin domains. The open conformation of this chromatin alleviates the spatial constraints at promoter sites and allows for targeted binding of transcription factors (Eberharter and Becker, 2002; Featherstone, 2002; Belmont, 2003). This correlative evidence seems to suggest that the virus recruits specific targeting or activation-related factors away from these potential transcription sites as a mechanism for global host RNAPII shut down.

With HAT recruitment, RNAPII shutdown and the total of highly acetylated epitopes globally decreased, questions remained as to the activity of HATs through HSV-1 infection. An enrichment of acetylated species was consistently detected by immunofluorescence at the periphery of nuclear compartments until very late in HSV-1 infection. This is a visible indicator that HAT activity persists through infection and that HDAC activity is likely not increased to account for the loss of transcription related acetylations. Although the sites of activity are redistributed through infection, the localization of activity in discrete locations within the nucleus parallels the functional compartmentalization in normal, uninfected cells. Although HATs may remain functionally competent, there is no HAT catalysis within viral compartments. The *in situ* HAT activity assay revealed a significant decrease of hyperacetylated H4 and H2B, a decrease mirrored in measured acetylation levels. Acetyltransferase activity is observed to increase for hyperacetylated H3 acetyl lysine epitopes in medium stages of infection. This may be due to the preferential recruitment of certain HATs with histone substrate selectivity (Carrozza and DeLuca, 1996; Utley *et al.*, 1998; Roth *et al.*, 2001). Its significance may lie in the determination of productive viral replication compartments (Kubat *et al.*, 2004), as it occurs at approximately the time of infection when viral DNA polymerase, an early gene (U_L30), is produced (Stingley *et al.*, 2000). More important to this discussion is that this acetyltransferase activity may be attributed to HAT associations with viral factors within distinct nuclear domains. The localization of this activity may contribute, either purposefully or inadvertently, to the global transcriptional shut-down of RNAPII on host genes.

HATs and HDACs exist in a dynamic equilibrium in close spatial proximity within the nucleus (Hendzel *et al.*, 1998; Yamagoe *et al.*, 2003). Therefore, it stands to reason that the recruitment of HATs to sites of viral replication, without a recruitment of HDACs, could account for the observed loss of highly acetylated species. This proves not to be the case as all HDACs and HDAC associated proteins are recruited to HSV-1 compartments. The direct interaction between HDAC1 and the viral immediate early protein ICP0 has been demonstrated for the bovine homolog but subsequent attempts to characterize a direct interaction in the human nucleus have been unsuccessful (Zhang and Jones, 2001; Poon *et al.*, 2003). This may be due to a spatial association through the reorganization and expansion of the intranuclear space, rather than a physical one. Our results showed the recruitment of the HDAC4 fragment consisting of a nuclear localization signal without the HDAC catalytic domain. In class I HDACs, the nuclear localization signal is contained within the catalytic domain, with the exception of HDAC2 (de Ruijter *et al.*, 2003). This gives support to a more generalized viral recruitment strategy whereby catalytic activity or sequence-specific recognition by viral factors may not predicate the recruitment of HDACs.

Although deacetylase activity may not be necessary within viral compartments for productive infection, the steady-state of HATs and HDACs favors deacetylation of chromatin through infection. Therefore, HDAC activity must persist. HDAC activity is generally not compromised through infection and therefore the net decrease in acetylation observed must be attributed to a change in the equilibrium in HAT and HDAC activities, compared to uninfected cells. The *in situ* HDAC assay confirms that for all dynamic HAT activity, there is a concurrent deacetylase activity. This is also consistent with the increased HAT activity for histone H3 observed at medium infection levels; this is matched by a coincident increase in HDAC activity. These spatially localized activities are the same property observed in normal cells with normal functional compartmentalization of HATs and HDACs. The global continuance of HDAC activity was confirmed by an *in vitro* HDAC assay, showing no significant decrease or inactivation of HDAC activity of isolated substrates until very late in viral infection. This matching of HAT and HDAC activities may suggest that a slight shift in equilibrium favoring deacetylase activity may arise from differential mobility of HATs and HDACs in the infected nucleus and the preferential recruitment of one or more HATs over HDACs.

4.2 A putative HSV-1 preferential recruitment mechanism, nuclear compartment dynamics and functional compartmentalization

When taken with bodies of data that suggest that class I acetyltransferase activity is a requirement for viral transcription (Preston and McFarlane, 1998; Poon *et al.*, 2003), recruitment may serve as a means to transcriptionally regulate the host cell, and in turn its own transcription, rather than be for the purposes of catalysis of substrate. The functional consequence of the recruitment of HATs has been established in this study whereas the means for recruitment and properties of recruited proteins in nuclear space must be explored. The mechanism through which early viral promoters are induced and greatly enhanced preferentially over host promoters remains unknown due to their identical sequence and lower copy numbers compared to that of host chromatin. The early recruitment of a small number of specific proteins may change the equilibrium and dynamics of transcriptionally relevant nuclear compartments. Furthermore, this may help in the recruitment of additional factors that usually assemble into larger coactivation or corepressor complexes, not through the formation of viable complexes *per se*, but through the relocation of favorable factor binding sites. This may be sufficient to tilt the equilibrium of factors needed for transcription, as well as elements of nuclear architecture, in favor of early viral promoters.

As nuclear compartments of an uninfected cell are dynamic, so too are viral replication compartments. Populations of CBP-GFP both within viral replication compartments or prereplicative foci are not immobilized or irreversibly bound to factors within the viral compartment. They are in constant flux and exchanging with the host chromatin, even during high levels of infection as host transcription is shut down. There also appears to be differential mobility of CBP-GFP between populations within viral compartments and that associating with the host chromatin. When photobleached, viral compartment populations take significantly more time to recover, demonstrating a more immobilized pool with a smaller effective diffusion coefficient. Similar results were obtained with other GFP constructs related to HATs that were tested, although no comparisons of diffusion rates were made. For enrichment to occur, viral compartments must contain a larger concentration of binding sites with a higher affinity for nuclear factors than that of binding sites in the host chromatin. If the binding affinity, and therefore smaller diffusion coefficient, of one or more HATs were greater than that of HDACs, the dynamic equilibrium would be shifted toward deacetylation of the host chromatin. How recruitment of factors occurs, and furthermore the preferential recruitment of CBP, remains to be examined.

Recruitment may occur through the sequence recognition of transcriptionally relevant factors by viral tegument proteins or those immediate early gene products that are expressed within minutes of infection (Homa *et al.*, 1988; Kibler *et al.*, 1991; Imbalzano and DeLuca, 1992; Cheung *et al.*, 1997). Considering the breadth of factors shown to be recruited, it is likely that a small number of factors are actively recruited in this way. For the cause of a possible initial recruitment event upon viral infection, factors that are introduced by the virus must be scrutinized. Histone acetyltransferase activity, transcriptional activation and chromatin structure have been linked to the potent transcriptional activator VP16, a tegument protein of HSV-1. Significant studies from the Belmont group have shown that VP16 activation correlates with acetyltransferase recruitment to an activated site near the gene promoter and increased localized acetyltransferase activity. This occurs through the sequential recruitment and assembly of chromatin remodeling complexes within condensed chromatin (Tumbar *et al.*, 1999; Memedula and Belmont, 2003). VP16 targeted to heterochromatic and centromeric regions is able to overcome position effect silencing through recruitment of the promoter to a more internal radial nuclear position (Tumbar and Belmont, 2001). VP16, surprisingly, shows little to no sequence-specific DNA binding activity. Rather, by directing the assembly of multiprotein-DNA transcriptional regulatory complexes, specificity and interpretation of cis-regulatory signals is achieved (Babb *et al.*, 2001). This is one of the first studies to analyze these aspects of transcription through the perturbation of normal transcription with the introduction of the intact virus and native VP16, rather than the artificial introduction of the activator to cells.

CBP is shown to be recruited to viral compartments very early in HSV-1 infection and before other histone acetyltransferases and deacetylases. Perhaps this initial recruitment is not surprising based upon its long-known association with VP16. VP16 is deposited in the nucleus with the viral genome, localizing within viral prereplicative foci. It is responsible for the first step in subversion of the host transcription machinery away from the host genome (Batterson and Roizman, 1983). Some populations of VP16 are associated with VP22, a protein targeted to PML bodies, and localizes later with the immediate early protein product ICP8 (Morrison *et al.*, 1998). VP16 contains an acidic activation domain (AAD) in its C-terminal region that has been shown to interact with the viral host shut-off protein (VHS; and later with ICP27), a tegument protein that acts downstream of initial viral transcription events to degrade host mRNA transcripts (Smibert *et al.*, 1994; Everly *et al.*, 2002). The AAD also interacts with components of transcriptional machinery (Stringer *et al.*, 1990; Goodrich *et al.*, 1993; Xiao *et al.*, 1994; Barlev *et al.*, 1995; Gupta *et al.*, 1996) and the histone acetyltransferases GCN5 and CBP/p300 *in vitro* and PCAF *in vivo* (Utley *et al.*, 1998; Tumbar *et al.*, 1999; Wang *et al.*, 2000b). Further to this, the AAD of VP16 forms an indiscriminate direct association *in vitro* and *in vivo* with both the amino and carboxy terminals of CBP (Ikeda *et al.*, 2002). The N-terminal domain of VP16 also has an indirect association with CBP through the large transcriptional coactivator complex Mediator in yeast and, through extension, activator-recruited cofactor in humans (Näär *et al.*, 2001; Ikeda *et al.*, 2002; Yang *et al.*, 2004). With at least four possible identified interactions of VP16 with CBP, the results concerning the preferential recruitment of CBP over other HATs and HDACs may be explained. Additionally, evidence suggests that the association of CBP with VP16 is nearly essential for the transactivation of immediate early genes. C-terminal truncations of VP16 that are severely retarded in IE gene expression are rescued by treatment with HDAC inhibitors, highlighting the necessity for acetylation, and possibly spatial targeting of acetylation, in productive viral infection (Preston and McFarlane, 1998; Mossman and Smiley, 1999; Yang *et al.*, 2002a; Poon *et al.*, 2003). The HDAC inhibitor TSA has also been shown to inhibit early and late gene transcription. This could indicate that VP16 may play a continuing role in competing with activated transcriptional machinery in order to target or sequester HATs for productive infection (Hobbs and DeLuca, 1999).

VP16 levels decrease through infection until transcribed as a late gene product for virion packaging. Microarray analysis of HSV-1 gene products conclude that VP16 produced through infection does not affect the viral kinetic cascade. The only significant action is the general activation of IE genes (Yang *et al.*, 2002a). Additionally, VP16 does not associate with HDAC1 or directly with PCAF (Ikeda *et al.*, 2002) and has not had any other reported associations with HDACs or HATs other than those mentioned above. Therefore, VP16 may be required for the initial recruitment of CBP over other factors related to transcription but it cannot account for the continuous recruitment of factors, including CBP, observed through the course of infection. Rather, preferential CBP recruitment to a concentration of high-affinity VP16 binding sites may be a nucleation event for RNAPII activity and formation of viral prereplication compartments. This may be sufficient to shift local acetylation dynamics, allowing for the transcription of immediate-early genes that enhance viral genome transcription and further recruit factors into a growing compartment.

This scheme of preferential recruitment may also apply to host transcriptional initiation in normal chromatin. It seems less likely that transcriptional complexes arrive at promoters preassembled. Rather, they are assembled where a nucleation event, like the binding of a coactivator to a promoter (Dundr *et al.*, 2002; Belmont, 2003). While some chromatin binding proteins are in constant flux, rapidly exchanging with the intranuclear pool, most exist bound to chromatin (Lever *et al.*, 2000; Cheutin *et al.*, 2003). This may also be true for transcriptional coactivators (T. Misteli, personal communication). Dependent upon the local protein environment and dynamics of factors like HATs, these proteins may be recruited into a coactivator complex, changing acetylation dynamics and transcription status. This organization affects the transcriptional landscape of the cell.

4.3 HSV-1 infection alters nuclear structural proteins in remodeling interchromatin space

Histone acetyltransferases and deacetylases, although controversial, are believed to be associated with the nuclear matrix. Therefore, the topic of a nuclear matrix and acetylation in transcriptional control through HSV-1 infection must be addressed. Evidence suggests that estrogen receptor dynamics in response to stimuli is dependent upon subnuclear localization of HATs and HDACs, intranuclear rearrangement, and association with elements of the nuclear matrix (Hager *et al.*, 2000; Stenoien *et al.*, 2000; Sun *et al.*, 2001). Recruitment to viral compartments, in whole or in part, may be associated with these conditions that are affected by HSV-1. The dual association of the estrogen receptor with HATs and HDACs strengthens the possibility of a larger global phenomenon involving the localization of HAT and HDAC activities and nuclear karyoskeletal arrangement in the transcriptional repression of host genes and concomitant activation of viral genes.

Several proteins tested for recruitment with both HATs and HDACs also have relationships with the nuclear matrix and are related to transcriptional control. mSin3A mediates the recruitment of corepressor complexes containing histone deacetylase activity to matrix attachment regions (MARs). The function of MARs is to provide a structural basis for the independent spatial and temporal regulation of gene expression and initiation. mSin3A recruitment is implicated in localized chromatin silencing by condensing chromatin structure (Stratling and Yu, 1999). Conversely, the mutated form of p53, an oncogenic mutation common in many tumors, may facilitate its functional activation by binding to MARs and causing the transactivation of genes through recruitment of transcriptional complexes (Will *et al.*, 1998). While specific functional relationships remain to be completely elucidated, retinoblastoma, ATM and topoisomerase II proteins are all associated with elements of the nuclear matrix (Mancini *et al.*, 1996; Smilenov *et al.*, 1999; Christensen *et al.*, 2002). Similar to estrogen receptor, it may be hypothesized that recruitment of these factors may have a causal relationship in the repression of host cell transcription. However, specific targeting of these individual factors becomes less likely as more associated proteins are found to be recruited to viral compartments. A global rearrangement of the nuclear matrix caused by, resulting from or unrelated to recruited histone acetyltransferase and deacetylase activities, may offer a more probable explanation. Our observations suggest that the establishment of functional complexes within viral compartments that are not related to viral transcription is highly unlikely. However, a rearrangement of the nuclear matrix may redistribute MARs to enrich in viral compartments. Alternatively, MARs may be disrupted, altering their positionally stable nuclear dynamics. This would allow factors to more freely diffuse and enrich in viral replication compartments as a result of steric accessibility, loss of host binding sites and putative relationships with other resident viral compartment proteins. Additionally, as the viral compartment remodels the interchromatin space and more HATs and HDACs are recruited to areas of chromatin depletion, host chromatin that is available to transcription factors and repressors is less abundant. These associated proteins may be recruited as a consequence of their inaccessibility to chromatin and therefore gradual functional inactivation.

The role of the nuclear matrix in the recruitment of the aforementioned proteins remains unclear. Evidence for nuclear matrix involvement in HSV-1 infection may come through the recruitment of other proteins that do not have a direct relationship with HATs and HDACs such as nuclear speckles (or IGCs) and the Nuclear Mitotic Apparatus (NuMA). Speckles are associated spatially with HAT and HDAC activity and are redistributed into different spatial arrangements upon infection. Interestingly, the splicing factor constituents seem to maintain a spatial relationship and a punctate pattern with CBP within large viral compartments of some cells. This relationship may be maintained for viral splicing events, as others have shown that the viral compartment itself is spatially organized (Arthur *et al.*, 1998; de Bruyn Kops *et al.*, 1998). Alternatively, the physical and dynamic properties of the proteins themselves may cause an association or mutual exclusion of factors within space that has no functional relevance.

NuMA, a nuclear scaffolding protein, has no apparent functional relationship with HATs or HDACs. Proponents of the nuclear matrix believe it is involved in the compartmentalization of both chromatin and interchromosomal functional domains and further determines the higher-order nuclear organization (Barboro *et al.*, 2003). Therefore, perturbation of nuclear organization through HSV-1 infection would surely affect, or be affected by, NuMA. Studies with two different types of viral infection revealed the proteolytic cleavage of NuMA at different times in infection (Taimen *et al.*, 2004). The difference in degradation pathways suggests that certain viruses, possibly including HSV-1, have virus-specific mechanisms for nuclear matrix deconstruction. The observation that the cleaved NuMA was excluded from condensed chromatin agrees with the recruitment of the protein to HSV-1 replication compartments. This finding suggests that viruses do facilitate the disassembly and/or redistribution of nuclear karyoskeletal elements. In addition, the immediate early protein ICP0, which has no known enzymatic activity, induces the degradation of the centromere protein CENP-C, illustrating another disruption in chromatin structural domains (Lomonte *et al.*, 2001). Alterations of structural proteins disrupt normal nuclear functional domains. It seems more likely that proteins with known associations to both the nuclear matrix and HATs and HDACs would lose their positionally stable binding sites. With increased nuclear mobility and a loss of binding sites to HATs and HDACs in their native domain, diffusion into and association with viral replication compartments seems a plausible function of nuclear matrix disruption at high levels of infection. Disruption of NuMA may also be in preparation for the expansion and lysis of the nucleus at very late stages of infection.

4.4 A model for HSV-1 infection: classical studies in virology meet the physical nature of the nucleus

Do protein compartments respond to the global transcriptional landscape or does the organization of protein compartments and nuclear scaffolding dictate the transcriptional landscape of a cell? The study of HSV-1 infection in the physical nucleus may hold clues to the nature of the organized and compartmentalized nucleus. Classic HSV-1 studies spanning thirty years have involved the characterization of the viral genome, viral replication and transcription, gene products and their interactions, *in vitro* associations with host proteins, and the spatial distribution of these proteins in nuclear space (Clements *et al.*, 1977; Batterson and Roizman, 1983; Puvion-Dutilleul *et al.*, 1985; Godowski and Knipe, 1986) . As a result, there is a considerable breadth of knowledge regarding the processes and players involved in viral processes while the state of the environment within which these processes occur, the host nucleus, has been largely ignored. Reconciliation of data from these two previously disparate fields of study should shed light on functional organization of the cell nucleus for both viral infection and host transcription.

The initial common thread linking nuclear architecture with viral replication is with promyelocytic leukemia (PML) bodies. These bodies were initially named ND10 (nuclear domain 10; also PODs), which are discrete interchromosomal accumulations of several proteins including PML, a putative component of nuclear architecture and thought to be antiviral defense centers (Maul, 1998). It had long been known that the sites for viral prereplicative foci in the nucleus showed punctate and non-random distribution (Clements *et al.*, 1977; Randall and Dinwoodie, 1986). Later, the non-random distribution of a population of prereplicative foci was attributed to the establishment at sites associated with PML bodies (Ishov and Maul, 1996; Maul *et al.*, 1996; Maul, 1998). This interaction and spatial distribution form replication territories around the PML body and have been characterized *in vivo* (Sourvinos and Everett, 2002). The PML-associated prereplicative sites subsequently destroy PML bodies, facilitated by the immediate early protein ICP0, within hours of infection to continue growing into productive viral compartments (Maul, 1998; Burkham *et al.*, 2001). I have discovered and recorded, in real time, the rapid kinetics and disruption of these normally positionally stable bodies early in HSV-1 infection using cells transiently transfected with PML-GFP (MacLean, unpublished observations). This disruption of PML may be due to an alteration of the nuclear matrix and allows the virus to further disrupt host nuclear processes by destroying nuclear organization. However, this still intimates that preexisting nuclear architecture and organization is essential for initial viral gene transcription.

The nature of the viral factors identified as being involved in the transcription of immediate early genes is exhaustive. However, these classical studies do not adequately explain the targeting of viral genomes to prereplicative sites, or whether or not they are associated with PML. A natural expansion and reinterpretation of these classical HSV-1 studies to include the spatial organization and physical nature of the nucleus may resolve this deficiency. The spatial distribution of viral transcription shows that replicative structures extend through the interior of the cell nucleus and appear to be spatially separate from the nuclear lamina (de Bruyn Kops and Knipe, 1994). PML bodies have a central radial distribution within the nucleus and are associated with permissive chromatin (Boisvert *et al.*, 2000; Borden, 2002). Therefore, a prerequisite for viral transcription may be a spatial association with permissive chromatin regions.

I propose that the process of immediate early gene transcription is a highly probabilistic process based largely on the diffusion or Brownian motion of the viral genome and its factors within nuclear space. Mobility within the nucleus has been reported as being heavily constrained due to macromolecular crowding caused by chromatin and non-chromatin nuclear compartments (Carmo-Fonseca *et al.*, 2002). Energy-dependent and molecular motor mechanisms have been proposed for various processes to overcome diffusional barriers. However, other reports argue that there is minimal macromolecular crowding by chromatin. Its volume occupies very little nuclear space compared to interchromatin territories (Cremer *et al.*, 2000). Mobility within the interchromatin space of macromolecular molecules may then vary dependent upon their size, shape and chemical properties in the nucleoplasm. DNA mobility in the nucleus is increasingly impeded as DNA length is increased (Lukacs *et al.*, 2000). The double stranded HSV-1 DNA duplex is 152 kilobase pairs in length (approximately 660 kDa). The mobility of DNA could be differentially impeded based on the genome being circularized or linear. Circularization decreases the effective size of the genome and releases it from the constraint of size-dependent diffusion (Seksek *et al.*, 1997; Lukacs *et al.*, 2000).

As the diffusion of the viral genome is not impeded, viral replication can theoretically be initiated anywhere within interchromatin space. However, not all prereplicative foci result in productive viral replication compartments. Productive compartment formation is dependent upon the spatial location of the immediate early transcription event. Establishment of prereplicative foci in these nuclear locations is a stochastic process. Viral capsids only contain one viral genome. Viral genomes are deposited into the nucleus in small numbers, dependent upon the multiplicity of infection. Individual cells infected with ICP0 null mutants below the threshold multiplicity have a high probability of establishing a nonproductive infection (Everett *et al.*, 2004a). This may be likely due to the decreased probability of a viral genome occupying the correct nuclear position for enriched transcriptional events when ICP0 is not able to associate with PML bodies and, due to this spatial disconnect, increased competition for transcription factors from the host nucleus. Figure 4.1 is a simplified diagram of a model I propose for productive (top series) and hindered or non-productive (bottom series) HSV-1 infection, highlighting histone acetylation and functional nuclear organization.

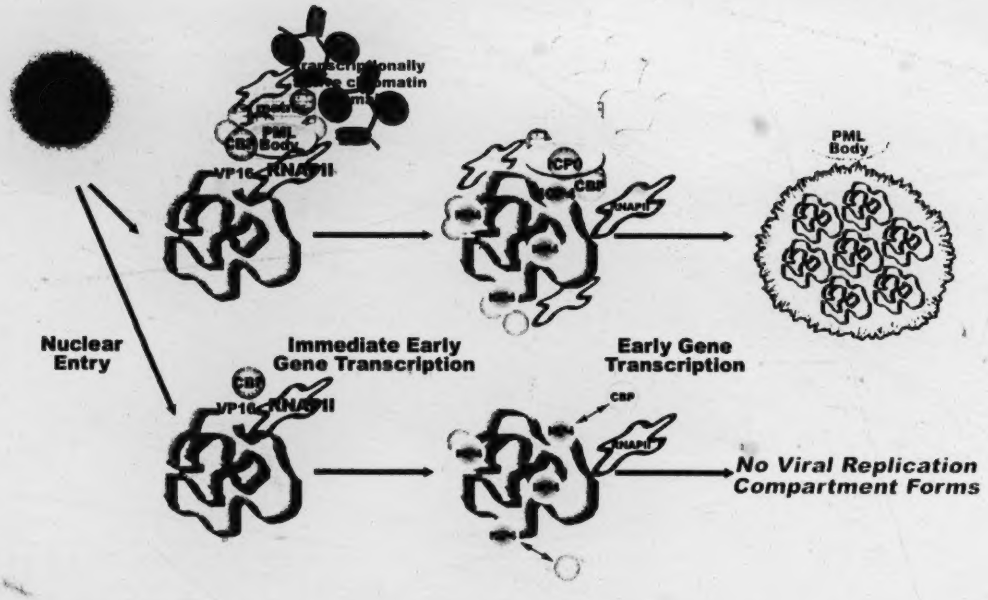

Figure 4.1 HSV-1 prereplicative compartments in permissive and non-permissive chromatin domains resulting in productive or unproductive viral replication compartments respectively. Top series: Upon nuclear entry of the viral genome (blue), VP16 (yellow) recruits CBP (green) enriched in PML bodies (pink) associated with nuclear matrix (gray) within permissive chromatin domains. RNAPII (green star) is recruited for IE gene transcription. ICP4 (red) transactivates the viral genome, recruiting factors away from the host chromatin. After early gene transcription, a viral replication compartments (starburst) is formed. PML and the nuclear matrix are destroyed and transcription of host chromatin is shut down (gray chromatin). Bottom series: VP16 recruits CBP and RNAPII from a smaller, more dynamic nuclear factor pool. After IE gene transcription, ICP4 attempts viral transactivation. The CBP pool is highly dynamic and productive interactions with RNAPII are fewer and less likely. The more stochastic and transient nature of the interaction fails to facilitate efficient transcription and no viral replication compartment forms in this region.

Upon entry into the nucleus, a viral genome may either establish itself in a region of permissive chromatin or condensed chromatin. Productive HSV-1 infections, as aforementioned, establish themselves in transcriptionally permissive chromatin domains. Viral tegument proteins are deposited into the nucleus with the viral genome. These proteins, whose actions are to target and recruit transcription factors, may be more accurately thought to locally concentrate relevant factors and change their regional dynamics. VP16 initiates transactivation of immediate early genes by binding, along with the cellular factors Oct1 and host cell factor (HCF), to the TAATGARAT elements present in all IE promoters the viral genome. This binding may occur in all viral genomes regardless of the viral genome's spatial location in the nucleus, most likely due to the initial proximity of VP16 to its preferential binding site. The recruitment of CBP to viral genomes may be through the VP16 acidic activation domain. This could be a nucleation event that recruits other transcription factors and aids in the assembly of the RNAPII holoenzyme (Memedula and Belmont, 2003).

Viral genomes that are associated with the functional architecture of PML bodies, and therefore the nuclear matrix, are successful at productive infections (Figure 4.1, top series(Maul *et al.*, 1996; Lukonis *et al.*, 1997; Sourvinos and Everett, 2002). The association with PML may have multiple purposes. First, CBP is enriched in PML bodies (Boisvert *et al.*, 2001). A shift in local steady-state concentrations of CBP in an area of actively transcribing chromatin would favor local deacetylation of the host chromatin. Transcription factors previously active on host promoters are recruited to the viral prereplicative site, perhaps as a direct result of CBP recruitment, and immediate early gene transcription commences. Secondly, the nuclear space around PML bodies is organized and is relatively positionally stable due to its association with the nuclear matrix. This may provide some positional stability to the viral genome and allow for interactions with the transcriptional machinery to continue within close proximity. This contributes to a less probabilistic transcription process for the initial transcription events.

Perpetuation of transcription and the establishment of viral replication compartments results from a combination of viral promoter enhancement by IE gene products, the interaction if IE gene products with functionally organized nuclear structures, and the subversion of host transcriptional machinery away from the host chromatin. In turn, these events alter existing functional compartments within the nucleus and contribute to tipping the tenuous balance between host and viral transcription. Immediate early gene products themselves distribute in specific spatial arrangements (de Bruyn Kops *et al.*, 1998). ICP4 colocalizes to prereplicative sites within minutes of infection (Randall and Dinwoodie, 1986). This is essential for the transactivation of early viral promoters that are sequentially identical to their host counterparts (Homa *et al.*, 1988). ICP4 persists in viral replication compartments and also transactivates late genes. I have found that ICP4 colocalizes with CBP, which may be a result of a previous recruitment by VP16 to viral genomes during the transcription of IE genes. ICP4 has also been implicated in recruiting transcriptional machinery and other HATs to prereplicative sites (Smith *et al.*, 1993; Carrozza and DeLuca, 1996), which can change local transcription factor dynamics by providing concentrated, high affinity binding sites. Others have found that ICP4 and ICP27, another IE gene product, are required for the targeting of PML bodies (Tang *et al.*, 2003; Everett *et al.*, 2004b). Viral infection may continue in the absence of IE gene products, but this is dose-dependent. Superinfection may increase the probability of a viral genome encountering a PML body (or concentrations of CBP in a nuclear compartment) and allow for meager transcription events to occur (Samaniego *et al.*, 1998).

Productive viral infections are severely impeded without the interaction of ICP4 with ICP0. ICP0 localizes with PML bodies and is responsible for their eventual disruption (Cheung *et al.*, 1997; Everett *et al.*, 2003). HSV-1 gene transcription is severely retarded with the deletion of the AAD in VP16 mutants (Mossman and Smiley, 1999). Viral transcription is also severely impeded in ICP4 null mutants (Samaniego *et al.*, 1998). In both cases, the lytic cascade can only be rescued by ICP0. ICP0 has also been associated with HDAC1 in bovine herpesvirus but does not affect the activity of HDAC1 (Zhang and Jones, 2001). Instead, it may be relevant to the localization of corepressor complexes to actively transcribing chromatin, further affecting the dynamic equilibrium of HATs and HDACs. ICP0-null mutants can undergo a great variety of fates, including quiescence, stalled infection at a variety of different stages, cell death, and, for a minor population, productive infection with an aberrant lytic cascade (Hobbs and DeLuca, 1999; Everett *et al.*, 2004a). Of these, cells with nonproductive infections can also express early gene products at low frequencies in a stochastic manner with severely delayed IE product expression (Everett *et al.*, 2004a). This suggests that ICP0, associating with cell architecture, is also responsible for directing viral gene transcription. Without this organizational structure, viral nucleoprotein structures would more freely diffuse, directed and dynamic alterations of functional cell compartments would not occur, and early viral replication events would be more stochastic and randomized as a result.

The degradation of PML (Figure 4.1; top series, right) and the alteration of the nuclear matrix may occur once viral replication compartments are fully established. The intranuclear space has expanded and factors relevant to HSV-1 transcription and replication are concentrated within viral replication compartments. Chromatin, at this point, is transcriptionally inactivated and the chromatin may be condensed, possibly due to the recruitment of HATs. This coincides roughly with the onset of viral genome replication, facilitated by the HSV-1 DNA polymerase transcribed from an early gene (Stingley *et al.*, 2000).

Where viral genomes locate to transcriptionally repressed chromatin regions, constituent factors of RNAPII holoenzymes are depleted and populations of CBP are more dynamic (Figure 4.1; bottom series). Therefore, the probability of a holoenzyme nucleation event occurring is significantly decreased. IE gene transcription is less effective without associations to areas that are rich in transcriptional factors. Immediate early gene products produced either after a long incubation of this genome or from another viral genome still create what are seen as prereplicative foci by ICP4. Again, the factors required for viral transcription, which exist in concentrated populations around PML bodies, are not in sufficient concentration to form a transcription complex. The more transient association with transcription factors and the lack of organized interchromatin space via nuclear matrix associations, or the virus's ability to destroy it, may make efficient viral replication less likely to occur. The process of transcription in the uninfected cell is similar; promoters kept away from transcriptionally associated nuclear compartments have more transiently associating factors, like CBP. Therefore, HAT acetylation cannot maintain permissive chromatin regions for a sustained period, making the chance of RNAPII nucleation and gene activation less likely.

Transcriptional regulation of host and viral genomes is a balancing exercise between viral and host factors, their interactions with each other, and their location in the nuclear space. The theory suggesting that the physical nature of the nucleus and its organization is a major determinant in whether or not productive infections occur may also have significance to the establishment of lytic or latent HSV-1 infections. One possible reason for the establishment of latency is the physical and transcriptional nature of the nuclear environment of the sensory neuron, where latent infections occur. As is the case in all classical HSV-1 studies, this concept has been previously overlooked in favor of studies analyzing protein products and their nuclear interactions. Neurons have a much higher transcriptional activity over that of other differentiated cell types. They also have a large nuclear volume along with greater euchromatic territories. Latent infections occur in specific sensory neurons where the viral genome is maintained intact (Jackson and DeLuca, 2003). However, the highly regulated productive cycle cascade of gene expression, characteristic of HSV infections, does not occur. Through infection of these neurons, lytic cycle reporter promoters' activity is detected in more than half of all infected cells after 24 hour incubation, and primarily localized at the site of entry of the virus. This is decreased over time in favor of LAT (latent gene) promoters (Arthur *et al.*, 2001). This highlights the importance of diffusion and genome position in the correct, permissive nuclear environment to allow HSV-1 infection and productive transcription. However, the increased competition with the transcribing cell for factor binding sites favors the host transcriptional architecture and organization.

PML bodies not appear to be involved in latent infections but that are required for lytic reactivation (Hsu and Everett, 2001). The difficulty in transcription of IE genes in a competitive environment hinders both the transcription of these genes and locally specific deposition of gene products to alter the equilibrium of cell processes. Differential regulation of viral gene transcription in neurons, including ICP0, seems to be tightly controlled by the cell rather than the virus (Loiacono *et al.*, 2002). Interestingly, a recent report found that LAT regions of viral genomes associated with acetylated histone H3 acetylated at lysines 9 and 14 (Kubat *et al.*, 2004). The relationship of the virus with medially acetylated histones may not be trivial and may also affect the mobility of viral genomes in cells. This highlights the effect of the nuclear environment on viral and host gene expression. It may also contribute to the maintenance of H3 acetylation at medium infection stages observed in this study.

The switch from latent to lytic transcription can be achieved with the treatment of the histone deacetylase inhibitor TSA. The inhibitor also induces IE promoter activation in latent infections of neuronal cells, which lack PML bodies, to trigger lytic replication (Arthur *et al.*, 2001). Latent HSV-1 DNA associates with nucleosomes while lytic viral DNA does not. Therefore, chromatin modification may be implicated in latent-lytic switching but may impede lytic expression of HSV-1 IE proteins by another unknown method, possibly through the increased potential for host cell transcription competing with VP16 gene transactivation. It may also implicate acetylated states of chromatin, and therefore its conformation, in transcriptional regulation of virus as it similarly regulates itself. Virus is out-competed and remains quiescent until there are changes to the transcriptional landscape, as is seen after treatment with TSA. Maintenance of the structural and functional organization of the nucleus is paramount to host transcriptional integrity.

4.5 Summary and future directions

In the cell nucleus, the organization of chromatin and the intranuclear space, the physical properties of the nucleus and dynamics of the factors within the cell are all functionally relevant. The controlled nuclear organization of HATs and HDACs, facilitated through nuclear architecture, is responsible for the regulation of histone acetylation and chromatin remodeling and, therefore, modulates transcription. This involves the steady-state recruitment of HATs and HDACs. While the majority of HATs and nuclear HDACs were examined in these studies, all known HATs and HDACs should be assayed for recruitment to viral compartments. Although there is strong evidence that CBP is the most likely candidate for preferential recruitment, it is possible that other HATs are also preferentially recruited and play a similar role in transcriptional shut down. The CBP-VP16 association should be characterized *in situ* or alternatively with Fluorescence Resonance Energy Transfer (FRET) studies *in vivo*. Since CBP may not be solely responsible for the preferential acetylation of H3 at medium stages of infection (Roth *et al.*, 2001), an *in vitro* HAT assay may further confirm that HAT activity is preserved through infection and that decreases in transcriptionally relevant acetylations are a result of recruitment. Transcription itself, although correlated with the decrease in relevant acetylation and HAT recruitment, should be examined temporally in conjunction with decreased acetylation.

I have shown that factors recruited to viral replication compartments are dynamic in nature and are in constant flux with regions outside of the viral compartment. Fluorescence Recovery After Photobleaching studies, particular those utilizing CBP-GFP, should be continued as a quantitative study to determine effective diffusion coefficients of virally recruited populations and populations associated with chromatin at different stages of infection. This would explore the relative influences of different molecular interactions on the steady-state distribution of HATs, HDACs and relate them to acetylation levels and loss of host transcription. It may also reveal a differential recruitment mechanism as infection progresses.

It is likely that the virus disrupts the steady-state distribution of functional host compartments through the active recruitment of factors required for viral transcription. The remainder of factors recruited through infection are likely those that have had their functional dynamics changed due to chromatin remodeling, a local depletion of relevant chromatin binding sites and perhaps a loss of the relevant factors needed for complex formation. However, nuclear architecture modifications, such as physical remodeling of the interchromatin space, have been implicated in this broad and continued recruitment through infection. Other studies have shown that functional nuclear compartmentalization, particularly in the case of PML bodies, is destroyed by the virus as a necessity for productive replication (Maul *et al.*, 1996; Lukonis and Weller, 1997; Sourvinos and Everett, 2002). PML bodies in and of themselves may not be sufficient for productive infections (Lopez *et al.*, 2002). Rather, those associated with disruption of PML bodies and the nuclear matrix are productive (Lukonis *et al.*, 1997). With the recruitment of NuMA, I have shown a link between infection and the alteration of the physical nuclear architecture. These studies characterizing the alteration of chromatin territories and nuclear architecture through infection are recent developments (Monier *et al.*, 2000). Following these changes *in vivo*, with GFP-tagged NuMA or other structural proteins, may further characterize functional alterations in nuclear space. Recent developments in the Belmont and Spector groups (Carpenter and Belmont, 2004; Janicki *et al.*, 2004) allowing gene transcription to be monitored in real time may be beneficial in our understanding of how the remodeling of nuclear architecture, and indeed alterations in steady-state compartment distributions, may affect host transcription.

This is one of the first studies to reconcile classical virology data with the physical properties and organization of the host nucleus. These are preliminary studies and theories concerning productive infection have been drawn from very disparate lines of experimentation. To further advance the knowledge of the process of HSV-1 infection, studies involving the diffusional nature of the viral genome and its timing of association with a number of key nuclear factors and viral factors should be undertaken. At least two systems to visualize HSV-1 genomes have been developed, one of which visualizes parental and replicated HSV-1 amplicon genomes (Sourvinos and Everett, 2002; Tanaka *et al.*, 2004). Additionally, HSV-1 systems have been genetically manipulated to express fluorescent protein-tagged gene products (Hutchinson *et al.*, 2002; Everett *et al.*, 2003). These systems are amenable to FRAP analysis and have the potential to reveal how the dynamics of genomes and their proteins in the nuclear environment influence the progression of HSV-1 infection. This could also solidify theoretical relationships between chromatin, nuclear factors and the process of transcriptional shut down through infection.

The body of knowledge about the nature of the nucleus - functional compartmentalization, constituent dynamics and structure – is rapidly expanding. Consolidation of existing genetic and evolving proteomic data with studies of the organization of the nucleus will assist in the elucidation of the process of transcription and other nuclear process.

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